Influence of the polyunsaturated fatty acids linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid on melanogenesis of B16 mouse melanoma cells and normal human melanocytes

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Dedicated to my wife Marlene

Contents

Abbreviations

AA	Arachidonic acid
ACTH	Adrenocorticotrophic hormone
ASP	Agouti signaling protein
bFGF	Basic fibroblast growth factor
cAMP	3',5'-cyclic adenosine monophosphate
DABCO	1,4-Diazabicyclo[2.2.2]octan
DHI	Dihydroxyindole
DHICA	5,6-Dihydroxyindole-2-carboxylic acid
DOPA	3,4-Dihydroxyphenylalanine
ET-x	Endothelin x (e. g. ET-1)
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GTP	Guanosine 5'-triphosphate
HGF	Hepatocyte growth factor
IBMX	3-Isobutyl-1-methylxanthine
ICAM-1	Intercellular adhesion molecule 1
IL-x	Interleukin x (e. g. IL-1)
INF-γ	Interferon-γ
IP_3	Inositol 1,4,5-trisphosphate
kDa	Kilo Dalton
LA	Linoleic acid
α-LA	α-Linolenic acid
γ-LA	γ-Linolenic acid
MGF	Mast cell growth factor
MHC	Major histocompatibility complex
MITF	Microphtalmia associated transcriprion factor
MSH	Melanocyte stimulating hormone
NHEK	Normal human epidermal keratinocytes
NHM	Normal human melanocytes
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PKA	cAMP dependent protein kinase
PKC-β	Protein kinase-β
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
POMC	Pro-opiomelanocortin
PUFA	Polyunsaturated fatty acid
TNF-α	Tumor necrosis factor-α
TRP1	Tyrosinase related protein 1
TRP2	Tyrosinase related protein 2
WFI	Water for injection

Summary

The influence of the polyunsaturated fatty acids (PUFA) linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid on pigmentation was examined using different *in vitro* models: monolayer cultures of B16 mouse melanoma cells, monolayer cultures of normal human melanocytes and a commercially available reconstructed pigmented epidermis that consists of normal, human-derived epidermal keratinocytes (NHEK) and melanocytes (NHM) which have been cultured to form a multilayered, highly differentiated model of the human epidermis that exhibits *in vivo*-like morphological and ultrastructural characteristics.

We used two different sublines of B16 origin called B16-F1 and B16-F10 for our experiments. In both sublines 25 μM of the PUFA listed above showed two distinct effects on melanogenesis of the cells: 1) the secretion of intracellularly produced melanin into the extracellular culture fluid was enhanced, and 2) the intracellular melanin content was decreased. The stimulation of melanin secretion was highest after an incubation time of 24 h, whereas the reduction of intracellular melanin was most effective after 72 h. α-Linolenic acid and γ-linolenic acid decreased intracellular melanin content more effectively than arachidonic acid and linoleic acid. Futhermore intracellular melanin content of murine melanoma cells was reduced more effectively in B16-F10 subline.

In both sublines tyrosinase activity was not affected, whereas tyrosinase content was reduced. Tyrosinase mRNA level of B16-F1 cells was decreased by linoleic and arachidonic acid. None of the fatty acids had an influence on tyrosinase mRNA levels of B16-F10 cells.

None of the polyunsaturated fatty acids at a concentration of 100 μM affected the melanogenesis of monolayer cultures of NHM when measuring the changes in mRNA level, content and activity of tyrosinase and in the amount of intracellular pigment. In addition the polyunsaturated fatty acids did not inhibit the pigmentation of the reconstructed pigmented epidermis.

1. Introduction

1.1 The skin

The skin has many functions: it not only protects the body against mechanical, thermic and chemical influences, but is also a highly sensitive organ for communication. Although the skin has only a thickness of a few millimeters, it is, with a surface of 1.8 m^2 and an average weight of 4.8 kilograms, one of the biggest organs of the human body. The skin represents an effective barrier and protects the remaining organs against mechanical, physical, chemical and biological damage. Furthermore, the skin possesses a crucial function in heat exchange and protection from loss of water. This transepidermal water loss is about 0.2 to 0.4 mg/cm^2/h at 30°C compared to the rate of evaporation of water from a free, uncovered water surface that is about 35 mg/cm^2/h at 30°C. The skin also has important endocrine functions such as the synthesis of vitamin D3, sex hormones and pheromones and it provides also immunological defenses.

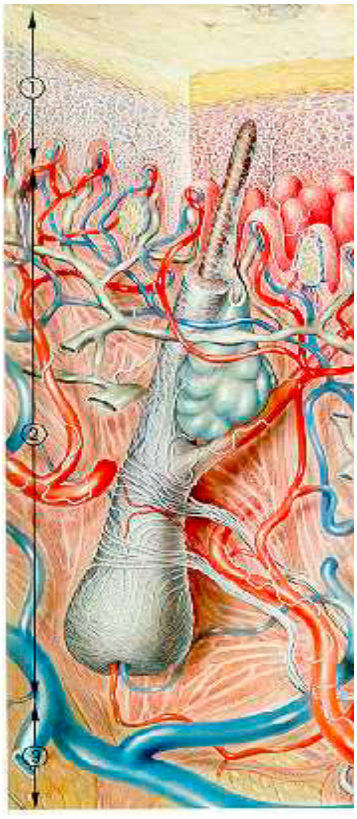


Fig. 1.1 Structure of the skin
The skin is divided into three layers: the epidermis (1), the dermis (2) and the hypodermis (3). The epidermis is a renewing stratified epithelium. The dermis, that consists of extracellular matrix (collagen, elastic fibers, etc.) and interspersed cells such as fibroblasts, is well supplied with blood vessels. There are no distinct borders between the dermis and the hypodermis that consists of fat cells (adipocytes).
(From "Elementa dermatologica"; Christophers E., Sterry W., Schubert Ch., Bräuer H.; Cassella-Riedel Pharma Frankfurt, 1987)

The skin is divided in three layers (**Fig. 1.1**): the epidermis arising from the ectoderm, the dermis and the hypodermis which are of mesodermal origin.

The epidermis is the outermost part of the skin. It is a continually renewing, laminated, squamous epithelium (**Fig. 1.2**). The main cells of the epidermis are the so-called keratinocytes. Beside the keratinocytes there are lymphocytes, Langerhans cells, melanocytes and Merkel cells interspersed among them. The keratinocytes are arranged in layers, which represent the different stages of their differentiation.

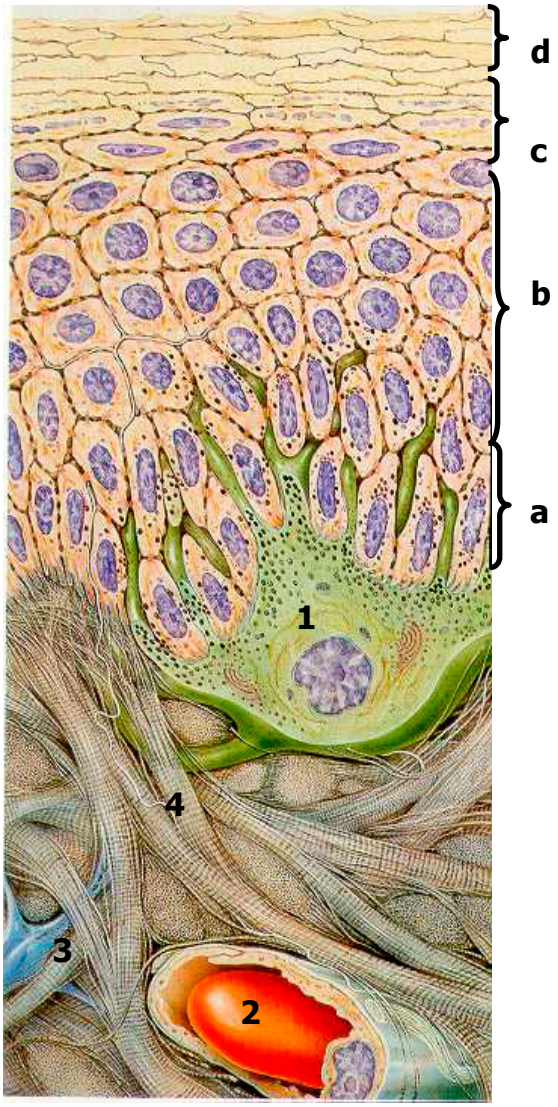


Fig. 1.2 Structure of epidermis and underlying dermis

The epidermis consists of layers of keratinocytes (a-d) that are named as follows:

stratum basale (a), stratum spinosum (b), stratum granulosum (c) and stratum corneum (d).

In the stratum basale pigment cells, the so-called melanocytes, are found (1).

The dermis is well supported by blood vessels (2) and consists of an extracellular matrix made of an amorphous ground substance and fibrillar structures such as collagen bundles (4). The dermis is a product of specialized cells called fibroblasts (3).

(From "Elementa dermatologica"; Christophers E., Sterry W., Schubert Ch., Bräuer H.; Cassella-Riedel Pharma Frankfurt, 1987)

The layers are named either according to their function or to their structure; their names are:

- Basal layer or stratum germinativum
- Spinous layer or stratum spinosum
- Granular layer or stratum granulosum
- Transitional layer or stratum lucidum
- Horny layer or stratum corneum

Some of the basal keratinocytes in the stratum germinativum are mitotically active and give rise to cells that move up to the more superficial layers. The stratum germinativum is followed by one or more layers of larger keratinocytes which are connected by desmosomes. This is the so called stratum spinosum. Its name derives from the spine-like appearance of the cells that result from shrinking during the processing of tissues for histological analyses.

In the stratum granulosum basophlic granulae can be seen in the light microscope, which contain keratohyalin, a precursor of keratin.

Above the stratum granulosum there is the stratum lucidum, which can be found only in very strongly cornified and hairless skin.

The stratum corneum is made of flattened epithelial cells (corneocytes) arranged in multiple layers. These layers are called keratinized layers because of the synthesis of the protein keratin in those cells. Keratin is a structure protein that is specific to the skin, hair and nails.

This layer of skin is, for the most part, dead. Acidic and basic keratins make up about 80% of the dry mass of the corneocytes.

It takes about one month from the time a basal cell leaves the bottom layer until it is desquamated.

The differentiation of keratinocytes includes several steps:

- Synthesis and modification of structural proteins, especially keratins
- Appearance of new organelles, reorganization of existing organelles, and loss of organelles
- Change in cell size and shape
- Specialization of cellular metabolism
- Changes in the properties of cell membranes
- Dehydration

The stratum corneum is an effective barrier against water loss and is mostly impermeable to external substances, such as drugs and toxic materials.

The dermis consists of extracellular matrix (collagen, elastic fibers, filamentous structures and amorphous ground substances made of glycosaminoglycans) that is interspersed with fibroblasts, macrophages, mast cells and lymphocytes.

The hypodermis consists of three fatty layers separated by connective tissue sheets. The primary functions of the hypodermis are thermoregulation, cushioning against mechanical trauma, contouring the body, filling space, and, the most important, serving as a readily available source of energy.

1.2 Melanocytes and melanogenesis

Although melanocytes comprise only a small proportion of the cells present in the epidermis of mammals, they are responsible for the production of the pigment melanin which accounts for virtually all of the visible pigmentation in their skin, hair and eyes. Melanin is produced in specific and unique subcellular organelles, the so-called melanosomes. The functions of melanin are still discussed. It has a clear role in camouflage and sexual display.

But this function is more important for animals than for *Homo sapiens.* It is thought that in man the main functions of melanin are protection against UV light radiation and scavenging of radicals.

1.2.1 Melanocytes

Melanocytes are highly dendritic cells that originate from the neural crest. During embryogenesis they migrate through the developing body to three principal locations: the skin, the eyes and the hair bulbs.

Melanocytes in the eye are distributed in the choroid, iris and retina. Eye melanocytes have very low rates of melanogenesis after fetal development and the synthesized pigment is not secreted, but remains in the melanocyte. Melanocytes in the eye are thought to function essentially as a photoprotective barrier.

Melanocytes that migrate to the skin are highly secretory. Within the hair bulbs the melanocytes produce melanosomes that are transferred to the hair shafts, thus giving the hair its visible color. The differences in hair color (and also in skin color) in man are caused by different types of melanin with differing visible colors that are produced by the melanocytes. The types of melanin produced and their distinct functional properties are discussed later in this chapter.

With age the melanocytes in the hair bulbs often become dormant and stop their melanin production, thus leading to the characteristic graying of the hair.

In skin itself the melanocytes are located at the junction of the dermis and the epidermis in the stratum germinativum. The melanocytes have an extremely low mitotic rate in contrast to the surrounding cells. The melanosomes produced in melanocytes are transferred to the neighboring keratinocytes. The exact mechanism of this transfer is still debated.

The association of the melanocyte and its surrounding keratinocytes has often been defined as the „epidermal melanin unit" where one melanocyte is normally associated with 36 keratinocytes.
As many of the precursors and intermediates in the melanin biosynthetic pathway are cytotoxic, the synthesis of the pigment takes place in specialized organelles named melanosomes. It seems that melanosomes are specialized members of the lysosomal lineage of organelles [1]. Melanosomes originate from the smooth endoplasmatic reticulum as a cytoplasmatic vesicle with an amorphous interior when analyzed by electron microscopy. At this point the granule is called a premelanosome and does not contain any of the enzymes needed for melanin synthesis. The main enzyme of melanogenesis, the so-called tyrosinase, and the other melanogenic determinants are synthesized on ribosomes. They are transported through the rough endoplasmatic reticulum to the Golgi apparatus where they are post-translationally processed and glycosylated. They are secreted within coated vesicles into the cytoplasmatic milieu and are transported specifically to premelanosomes. The enzymes are integrated in the melanosomal membrane with their catalytic centers facing inward. During this process the amorphous structure of the melanosome changes into a characteristic fibrilar pattern along the longitudinal axis of the melanosome. Studies have shown that tyrosinase is catalytically competent while in transit through the Golgi apparatus. It is not clear how melanin synthesis is delayed until the enzyme arrives at the melanosome. It has been proposed that specific inhibitors are responsible for preventing the synthesis of melanin until incorporation within the melanosome. There are four stages in the maturation of a melanosome: stage I, the "premelanosome" is a spherical organelle with matrix filaments that are not well defined; stage II, in which the typical elliptical melanosome is filled with a filamentous or laminar matrix; stage III is characterized by the deposition of electron dense material on this matrix; and stage IV, with complete opacification of melanosomal contents by melanin deposition [1].

As this process of maturation of the melanosome proceeds, the organelle is steadily moving away from the perinuclear region near the Golgi apparatus to the peripheral dendritic areas. Recent studies showed that the actin-based molecular motor Myosin V together with the protein Rab27a binds to melanosomes and participates in their transport to dendrites [2]. It was also shown that kinesin plays an important role in melanosome transfer. It is speculated that Myosin V together with Rab 27a is responsible for the transport of melanosomes in the cell cortex, while kinesin drives the melanosome transfer in the perinuclear region.

As mature melanosomes arrive at the end of the melanocyte dendrite, they are secreted in areas where the melanocytes intercalate with keratinocytes. The actual transfer of melanosomes into keratinocytes and the keratinocyte-melanocyte interactions during the transfer are not well characterized. Early light and electron microscopy studies suggested numerous possible mechanisms for melanosome transfer [3]. These include the release of melanosomes into the extracellular space followed by endocytosis, direct inoculation („injection"), keratinocyte-melanocyte membrane fusion and phagocytosis. Recent studies showed that the protease-activated receptor-2 (PAR-2) and lectins and their glycoconjugates play an important role in melanosome transfer [4].

Once melanosomes are transferred to keratinocytes, they are packaged in secondary lysosomes and arranged as supranuclear melanin caps above the keratinocyte nuclei [5]. As keratinocytes ascend to the epidermal surface from the basal and suprabasal layers where melanosome transfer takes place, melanosomes also ascend and are retained in the horny-layer cells for approximately two weeks. In Caucasian skin melanosomes show marked degradation and appear as melanin dust. In contrast, melanosomes from dark-skinned Negroid people show little degradation in the horny-layer cells [6].

1.2.2 Melanin biosynthesis

There are basically two distinct forms of melanin in mammals. One group is the so-called eumelanins that are brown to black and insoluble. The other group is the pheomelanins that are reddish-brown and soluble in alkali. The initial steps in the synthesis of eumelanin and pheomelanin are controlled by the enzyme tyrosinase, which oxidizes the amino acid tyrosine to 3,4-dihydroxyphenylalanine (DOPA) (see **Fig. 1.3**).

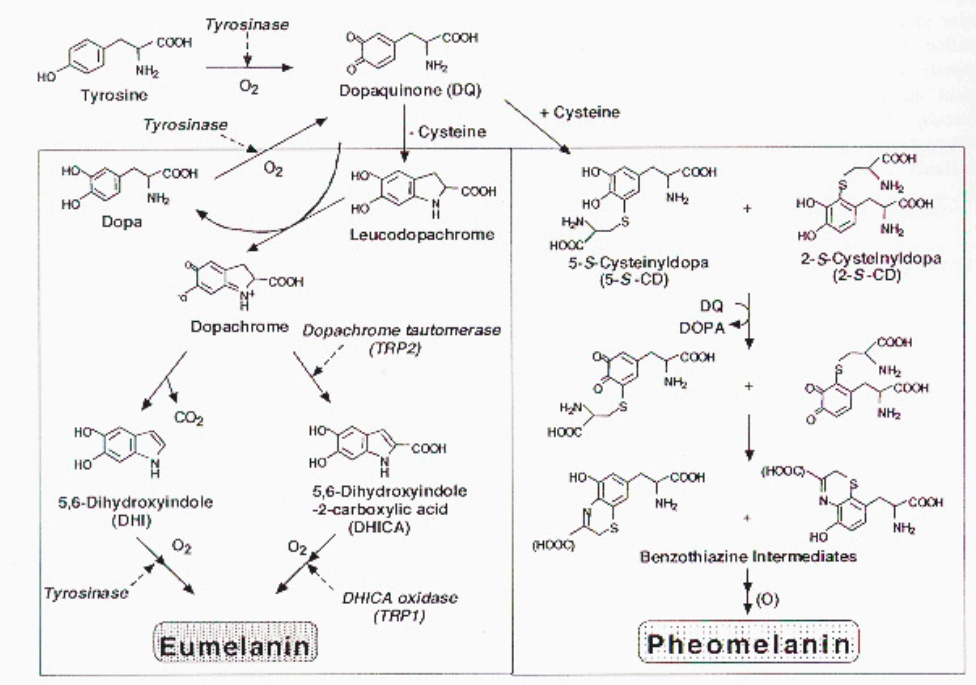


Fig. 1.3 The biosynthetic pathway of eumelanin and pheomelanin. (From Ito et al. [7])

DOPA therefore can spontaneously autooxidize to dopaquinone without tyrosinase, but at slower rates than in presence of the enzyme. Dopaquinone is an extremely reactive compound that, in the absence of thiols in the reaction medium, undergoes intramolecular cyclization leading to leukodopachrome and then to dopachrome. Dopachrome decarboxylates spontaneously to dihydroxyindole (DHI).

In the presence of divalent cations and the enzyme DOPAchrome tautomerase, also called tyrosinase related protein 2 (TRP2), the intermediate 5,6-dihydroxyindole-2-carboxylic acid (DHICA) will result. DHI is oxidized to indole-5,6-quinone and DHICA is oxidized to Indole-5,6-quinone-carboxylic acid. It is speculated that the oxidation of DHICA is catalyzed by an enzyme called DHICA oxidase which is synonymous with tyrosinase related protein 1 (TRP1) and the oxidation of DHI by tyrosinase. The quinones are thought to build melanin by oxidative polymerization. Whether this polymerization step is under enzymatic control is not yet clear. It is thought that peroxidase or the melanocyte specific protein Pmel-17 play a role in this step of melanin synthesis [8].

Melanins generated *in vitro* from DHICA are brown in color, poorly soluble and of intermediate weight, whereas those generated from DHI are black, totally insoluble and of high molecular weight. These melanins are termed eumelanins. Eumelanins are a mixture of DHI- and DHICA melanins and on the individual basis, the chemistry of these pigments may vary to a considerable extent. Thus various forms of eumelanin that can be found in human skin interact differently with UV light.

The other major diversion in the melanin biosynthesis pathway occurs upstream in the pathway immediately following the conversion of DOPA to dopaquinone. In the presence of sulfhydryl donors, probably cysteine, dopaquinone is converted to cysteinyl-DOPA.

Further oxidation, cyclization and polymerization leads to the formation of pheomelanin. Pheomelanins have a yellowish-red color, are soluble in alkali and have a low molecular weight.

These different types of melanin are responsible for the differences in hair color in mammals and in man. Yellow to bright red hair result from the production of pheomelanin, whereas brownish to black hair have their origin in eumelanin production.

It is not clear, how the switch from eumelanin- to pheomelanin production or vice versa is controlled .

For mice it is known that the interactions of melanocyte stimulating hormone (MSH) and agouti signaling protein (ASP) play an important role [9, 10]. A black phenotype, originated by eumelanin production is elicited at conditions under which there is an overstimulation of the MSH receptor, whereas in contrast, conditions under which the function of the MSH receptor is abrogated or blocked by ASP result in the production of pheomelanin leading to a yellowish phenotype. Today it is known that MSH and ASP function similarly in humans. It was shown that the phenotype of red hair and fair skin unable to tan is associated with mutations in the gene for the melanocyte-stimulating hormone receptor [11].

One can put the question why there exist different types of melanin. Every type of melanin has its own physical and biological characteristics. DHI melanin is very good in photoabsorption, shows no phototoxicity, but is highly cytotoxic. DHICA-melanins have reduced photabsorption, no phototoxicity and are less cytotoxic. In contrast, pheomelanin provides only little photoabsorption, has a high phototoxic potential, but low cytotoxicity. The optimum type of melanin must be a compromise between photoprotection while minimizing cyto- and phototoxicity.

1.2.3 Regulation of melanogenesis

Mammalian and therefore human pigmentation is a very complex process. This biosynthetic pathway is regulated at different levels by a variety of distinct factors. There are enzymes such as tyrosinase, TRP1 and TRP2 that regulate the synthesis of melanin.

These enzymes are also regulated (their activity, their production at the transcriptional and translational level, etc.). Keratinocytes produce a lot of substances that play an important role in regulating melanocyte growth and differentiation. One of the major factors that affect human pigmentation is UV radiation.

One can divide skin pigmentation in two components. The first, constitutive pigmentation, is the pigmentation which is genetically determined in the absence of stimulatory influences, or in other words, the level of pigmentation in parts of the body that are not normally exposed to UV radiation. The second component, facultative pigmentation, is the level of pigmentation or tanning that occurs in response to stimulatory effects that are in man UV radiation and, to a certain degree, hormones.
These external stimuli are converted in intracellular messenger molecules that affect melanogenesis. Different signaling pathways mediating melanogenesis will be discussed later in this chapter.

1.2.3.1 Enzymes in skin pigmentation

There are three key enzymes known to date, all physiologically associated with melanosomes: tyrosinase, TRP1 and TRP2 (**Tab. 1.1**). Tyrosinase, TRP1 and TRP2 are glycoproteins embedded in the melanosome membrane that share 70-80% nucleotide sequence homology with 30-40% amino acid identity. Among these three enzymes, tyrosinase is the most critical and rate limiting enzyme.

Tyrosinase

Tyrosinase is the rate-limiting enzyme in the melanin biosynthesis pathway. It is a multifunctional copper-containing glycoprotein with a molecular weight *de novo* of 65 kDa and about 75 kDa when glycosylated [12]. It is an unusual enzyme as it catalyzes three distinct chemical reactions [13].
It catalyzes the first two steps in melanogenesis: the hydroxylation of tyrosine to DOPA and then the oxidation of DOPA to dopaquinone. The third catalytic activity is the oxidation of DHI to indole-5,6-quinone. This function of the enzyme is still debated.

Another curiosity of tyrosinase is the fact that it requires DOPA as cofactor for the tyrosine hydroxylase reaction [14]. The product is therefore the cofactor for the synthesis of the product.

Rates of tyrosine hydroxylation in the absence of the cofactor are negligible. That raises the question where the initial DOPA cofactor derives from, since DOPA is not a normal amino acid available within the cell. This important question is still unresolved.

Tyrosinase is an extremely stable protein that is highly resistant to heat or proteases. It also has an unusually long biological half-life up to 10 hours *in vivo.*

Tyrosinase can be divided into three domains: an inner domain that resides inside the melanosome, a transmembrane domain and a cytoplasmatic domain. The biggest part of the enzyme resides inside the melanosome and only 10% or 30 amino acids constitute to the cytoplasmatic domain [15]. The inner domain contains the whole catalytic activity while the cytoplasmatic domain seems to play an important role in cellular trafficking of tyrosinase and in regulation of tyrosinase activity.

The transcription of the tyrosinase gene is regulated by a transcription factor called microphtalmia-associated transcription factor (MITF) [16]. MITF belongs to the basic-helix-loop-helix-zipper family and is known to interact with a specific DNA sequence termed M-box with the sequence GTCATGTGGCT that is present in the promoter region of the tyrosinase gene [17]. There is evidence that the intracellular second messenger cAMP increases tyrosinase gene expression by enhancing the interaction between MITF and the M-box [18].

The regulation of the activity of tyrosinase is managed by phosphorylation of the enzyme by protein kinase C-β [19].

The cytoplasmatic domain of tyrosinase contains two serine residues at amino acid positions 505 and 509 that are candidates for phosphorylation by this serine/threonine kinase [15].

A recent study showed that PKC-β phosphorylates both serines but it is not clear whether phosphorylation of one or both of the serine residues is required for the activation of tyrosinase [20].

TRP1

The function of TRP1 is not clear yet. It has been proposed to be a lower specific activity tyrosinase [21], a dopachrome tautomerase [22], a tyrosine hydroxylase [23], a DOPA oxidase [24], a melanosomal catalase [25], or a DHICA oxidase [8]. More recently it has been suggested that TRP1 influences the activity of tyrosinase by stabilizing and/or forming a complex with tyrosinase [26]. In the protein data base Swiss Prot the catalytic function of TRP1 is stated as „Oxidation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) into Indole-5,6-quinone-2-carboxylic acid. May regulate or influence the type of melanin synthesized". The molecular weight of the precursor molecule is about 60 kDa. Synonyms for the enzyme are: DHICA oxidase, catalase B, glycoprotein 75 and melanoma antigen gp75. About the regulation of the transcription of the corresponding gene and the activity of the enzyme nothing is known.

TRP2

The function of TRP2 is that of a dopachrome tautomerase [27]. The molecular weight of the enzyme is disputed. The records range from 46 to 80 kDa.

The reason for these discrepancies in molecular weight is the fact that it is very difficult to purify TRP2 as it co-purifies with tyrosinase and may exist in a complex with tyrosinase, TRP1 and possibly other proteins.

	Tyrosinase	TRP1	TRP2
Synonym	Monophenol monooxy-genase	DHICA oxidase Catalase B Glycoprotein-75 Melanoma antigen gp75	Dopachrome tautomerase Dopachrome delta-isomerase
Specificity	Melanocyte	Melanocyte	Melanocyte
Molecular weight	65 kDa (*de novo*) 75 kDa (glycosylated)	60 kDa (unprocessed precursor)	46 – 80 kDa (in dispute)
Isoelectric point	4.3	Unknown	Unknown
Catalytic activity	Tyrosine → DOPA DOPA → Dopaquinone DHI → Indolquinone	DHICA →Indolquinone carboxylic acid	Dopachrome → DHICA
Half life	4 – 10 h (*in vivo*)	Unknown	Unknown
Miscellaneous	Heat-stable Protease-stable Chelator-sensitive Glycosylated Membrane-bound DOPA cofactor-dependent	Glycosylated Membrane-bound	Heat-sensitive Protease-sensitive Chelator-insensitive Glycosylated Membrane bound DOPA cofactor-independent

Table 1.1 Characteristics of melanogenic enzymes

1.2.3.2 Paracrine control

Keratinocytes play an important role in the regulation of melanocyte growth and differentiation. They produce a variety of different factors that act on melanocytes.

Melanocytes do not produce their own growth factors under normal conditions. There exist reports that one of the first steps in melanoma development is the synthesis of autocrine growth factor basic fibroblast growth factor (bFGF) by melanocytes [28]. There is also evidence that α-MSH acts as an autocrine factor on melanocytes in melanogenesis [29].

Under normal conditions the proliferation of melanocytes is regulated by keratinocytes through the production of the main melanocyte growth factor bFGF. Other melanocyte growth factors include mast cell growth factor (MGF) and hepatocyte growth factor (HGF).

Their actions are not specific to melanocytes. HGF stimulates the growth of a variety of epithelial cells, and mast cell growth factor (synonymous with stem cell factor, steel factor or c-kit ligand) affects the proliferation of numerous cell types.

The most studied of the locally produced factors that regulate melanocytes are the cytokines and other inflammatory mediators. It has been suspected that some of these substances mediate the effects of UV radiation and post-inflammatory pigmentary responses.

The cytokines interleukin 1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) have been reported to inhibit both melanogenesis and melanocyte proliferation. These cytokines as well as TNF-β, IL-7 and interferon-γ (INF-γ) also induce the expression of intercellular adhesion molecule-1 (ICAM-1) and MHC class II molecules on cultured melanocytes. This could reflect a change in melanocyte function from a melanin producing cell to an immune competent cell.

Endothelins play an important role in melanocyte development, growth, melanogenesis, motility and dendricity. Studies showed that ET-1 affects melanocyte dendricity [30], proliferation [31], and melanogenesis [32], while ET-3 plays an important part in regulation of progenitor number and differentiation in melanocyte development [33]. To date ET-1 is the only mitogen that increases both melanin synthesis and melanocyte proliferation at very low doses in the nanomolar range [32].

1.2.3.3 Hormonal control

Melanocytes respond to many hormonal stimuli, and the two most important groups are MSH peptides and sex steroids.

MSH peptides

There exist several MSH peptides. They are formed together with numerous other peptides including adrenocorticotrophic hormone

(ACTH), lipotrophins and endorphins from a precursor molecule called pro-opiomelanocortin (POMC) (see **Fig. 1.4**).

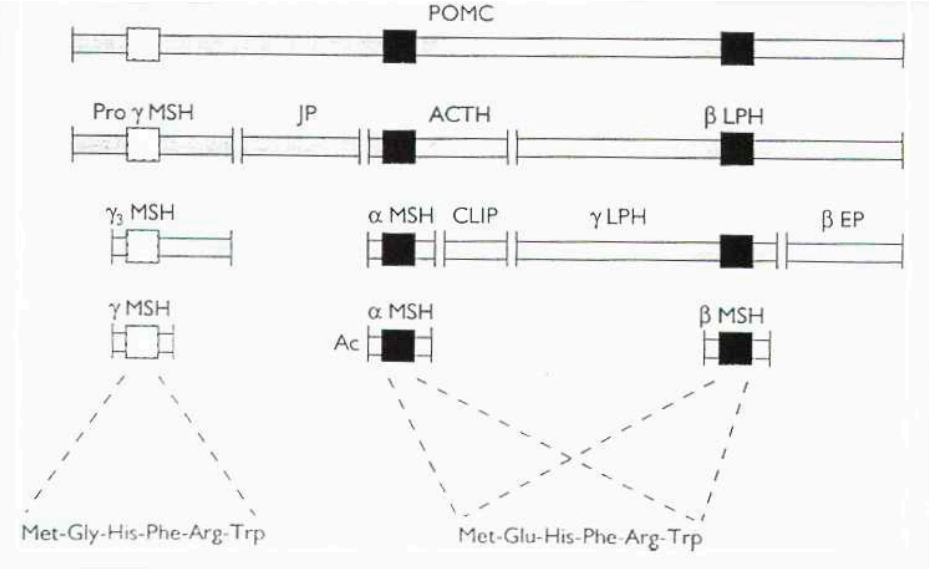


Fig. 1.4 The POMC family of peptides (from Thody [12])
The core sequences are shown as ■ and as □.
ACTH – adrenocorticotrophic hormone, CLIP – corticotrophic like intermediate lobe peptide, βEP – β-endorphin, JP – joining peptide, LPH – lipotrophin, MSH – melanocyte stimulating hormone, POMC – pro-opiomelanocortin

The main site of POMC production is the pituitary gland but there is evidence that this protein and its cleavage products are also produced in the skin [34, 35]. The pigmentary action of α-MSH in man was first demonstrated by Lerner and McGuire [36].

There exist several reports that α-MSH and related peptides fail to have an effect on melanogenesis in cultured human melanocytes [37]. There is strong evidence that this unresponsiveness of cultured melanocytes to MSH *in vitro* is related to mitogens normally used in melanocyte cell culture. If these mitogens are avoided, human melanocytes readily respond to MSH [38].

In mice the main effect of MSH is the increase of synthesis of eumelanin.

From the MSH or melanocortin receptor seven different subtypes are known to date. Melanocytes express melanocortin receptor 1 (MC1R) [39].

The MC1R is a seven pass transmembrane G protein coupled receptor which activation leads to an increase in the second messenger cAMP. In man at least 20 allelic variants of the MC1R have been described to date [11]. Four alleles are clear loss-of-function mutations that are causally associated with the development of red hair and cutaneous sensitivity to UV radiation (tendency to burn) that is recognized as a major risk factor for melanoma skin cancer.

Recent studies showed that α-MSH is not the only POMC peptide with melanogenic activity and it is now established that ACTH binds to the MC1R and is also active in this respect [40]. There are reports that ACTH peptides are even more potent than α-MSH in stimulating melanogenesis and that ACTH1-17 is especially potent [29].

As it was said before, the major site of α-MSH production in mammals is the intermediate lobe of the pituitary. However this lobe is vestigial in adult humans and little if any α-MSH is produced there. Now it is known that the molecule is produced in several cell types in the skin including keratinocytes and melanocytes and therefore the action that α-MSH has in the skin, is mediated by paracrine and/or autocrine mechanisms. The same applies to ACTH as it is produced by human keratinocytes.

Therefore these peptides are not hormones in the sense of the definition of a hormone.

Steroids

Increased pigmentation of the nipples, areola, face, abdominal skin and genitalia is described for pregnant women. A study showed that sex steroids (diethylstilbestrol and estradiol) lead to a 1.2 to 2.5 fold increase of tyrosinase transcripts in cultured human melanocytes [41].

The amount of TRP1 transcripts was likewise enhanced but TRP2 transcripts were upregulated of about twenty fold over the initial transcript level.
This is a strong evidence that hyperpigmentation during pregnancy is mediated by a direct induction of melanogenesis by sex steroids.

1.2.3.4 Transmembrane and intracellular pathways

Several different signal transduction pathways operate in pigment cells, the best known being the adenylate cyclase/cAMP system that is activated by POMC derived peptides. The intracellular level of cAMP can also be upregulated by drugs or chemicals such as cholera toxin or isobutylmethyl xanthine (IBMX). It was shown that the action of cAMP is mediated by a cAMP-dependent protein kinase (PKA) which is a serine/threonine kinase. Activation of the cAMP pathway leads to an increase in tyrosinase gene transcription by enhancing the interaction of the specific transcription factor MITF with a specific DNA sequence named M-Box that is located in the promoter region of the tyrosinase gene.
The second important pathway in human melanogenesis is the protein kinase C (PKC) pathway. In the last years experiments have shown that PKC-β is necessary to activate tyrosinase [20].
Using a pigmented human melanoma cell line that expresses PKC-β and an amelanotic subclone that has lost the expression of PKC-β but has equally abundant tyrosinase protein, it was shown that transfection of PKC-β cDNA into amelanotic subclone cells activated tyrosinase.
It is thought that PCK-β activates tyrosinase by phosphorylation of the enzyme at the cytoplasmatic tail and also that the inactive form of PKC-β is activated through the intracellular messenger diacylglycerol (DAG).

DAG is produced together with 1,4,5-trisphosphate (IP_3) from the precursor molecule phosphatidylinositol 4,5-bisphosphate (PIP_2) that is cleaved by phospholipase C (PLC). IP_3 interacts with IP_3-sensitive Ca^{2+} channels in the endoplasmatic reticulum, causing release of stored Ca^{2+} that bind to calmodulin and activate a calmodulin dependent kinase (CaM kinase).

PLC can be activated by activated G proteins or by receptor tyrosine kinases that are discussed later in this chapter. To date it is known that ET-1 acts through the PLC/PKC pathway. But it is speculated, that the different signal transduction pathways are not isolated but that there exists a cross-talk between them. These speculations are supported by findings that ET-1 increases not only tyrosinase activity but also the intracellular cAMP level and the tyrosinase gene transcription.

The third important pathway is the tyrosine kinase pathway.

Receptor tyrosine kinases (RTKs) form a large and important class of cell-surface receptors whose ligands are soluble or membrane-bound peptide/protein hormones including insulin and growth factors. In the skin, growth factors act not like hormones in the definition of a hormone, they act as paracrine or autocrine factors. To date it is known that the growth factors b-FGF, MGF and HGF bind to tyrosine kinase receptors on the surface on human melanocytes [42].

Binding of the ligands to the receptor causes the RTK to dimerize and the protein kinase of each receptor monomer then phosphorylates a distinct set of tyrosine residues in the cytosolic domain of its dimer partner. This process is termed autophosphorylation.

A protein called Ras that belongs to the GTPase superfamily associates with the activated domain of the RTK. Ras is a kind of an intracellular switch as it is in the on position or in its active form when GTP is bound, and in the off position or in the inactive form when GTP is hydrolyzed to GDP. The activated Ras binds to the N-terminal domain of a serine/threonine kinase called Raf. Raf binds to MEK and phosphorylates it.

MEK is a protein kinase that has a dual specificity as it phosphorylates both tyrosine and serine residues. MEK phosphorylates and activates MAP kinase that is another serine/threonine kinase. Activated MAP kinase translocates to the nucleus and phosphorylates the ternary complex factors, which initiates transcription of genes such as c-fos that is a very important transcription factor.

1.2.3.5 Effect of UV radiation

In man, sunlight is the most important physiological stimulator of the pigmentary system and is responsible for the tanning response. It is the UV spectrum of solar radiation that causes the tanning response in the skin. UV radiation can be divided into UVC (200-290 nm), UVB (290-320 nm) and UVA (320-380 nm). UVC is absorbed in the atmosphere and does not reach the Earth's surface. UVA and UVB have different effects on human pigmentation. It is thought that UVA is responsible for the immediate tanning that occurs within 24 hours and is based on oxidation of pre-existing melanin [43] and redistribution of melanosomes in the melanocyte from a perinuclear position to a more peripheral, dendritic distribution [44]. Immediate tanning is not very photoprotective against subsequent UV injury in contrast to the delayed tanning response.

The delayed tanning response, that begins as the immediate response fades out and progresses for at least 3-5 days after UV exposure, can be induced by both UVA and UVB, although the UVA induced response is of 2-3 orders of magnitude less efficient.

The delayed tanning response is accompanied by an increase in tyrosinase activity [45]. UVA induced delayed tanning requires oxygen at the time of irradiation, whereas the UVB response does not.

It is thought that UV-induced DNA photodamage and/or its repair is at least one of the initial signals in stimulation of melanogenesis due to UV radiation [46].

Other signals are paracrine/autocrine factors that are produced by keratinocytes or melanocytes after UV stimulation.

1.3 Fatty acids

1.3.1 Nomenclature of fatty acids

Fatty acids are monocarboxylic acids with an acyclic and unbranched structure. Their basic formula is $CH_3[CH_2]_nCOOH$ where n can be any number from two to twentytwo. The total number of carbon atoms is in most cases even although odd-numbered carbon atoms containing fatty acids also exist.

Fatty acids can be classified according to their chain length and also according to their double bonds. Fatty acids that contain no double bonds are called saturated, with one double bond they are named monounsaturated and with two or more double bonds polyunsaturated. Depending on the number of double bonds from 2, 3, 4, 5 and 6 they are named dienoic, trienoic, tetraenoic, pentaenoic, and hexaneoic.

The carbon atoms of the fatty acid can be numbered from carboxyl group to the terminal methyl group (Δ numbering system) or from the methyl group to the carboxyl side (W or n numbering system).

Palmitic acid, that is a saturated fatty acid, is abbreviated as C_{16}:0. Palmitoleic acid that contains one double bond is abbreviated as C_{16}:1,Δ_9 or as C_{16}:1,n-7 depending on the numbering system that is used.

The number after the Δ or n signifies the position of the double bond relative to the carboxyl group or to the terminal methyl group, respectively.

1.3.2 Biosynthesis of polyunsaturated fatty acids

Mammalian tissues contain four series of PUFA: n-9, n-7, n-6 and n-3 [47]. Interconversion between these families is not possible. The precursors of two of these groups are the monounsaturated fatty acids palmitolieic and oleic acids, which can be synthesized endogenously from saturated fatty acids. The precursors for the other two groups are necessarily derived from dietary sources: linoleic and linolenic acid. All of the PUFAs found in mammalian tissues are synthesized from these four precursors by desaturation and elongation catalyzed by enzymes called desaturases and elongases.

1.3.3 PUFA in cutaneous biology

From a series of papers published in 1929 and 1930 it was speculated that warm-blooded animals are not able to synthesize appreciable quantities of certain fatty acids [48, 49]. Rats maintained on a fat-free diet over a long period developed abnormalities such as growth retardation, severe scaly dermatosis and extensive water loss through the skin. These deficiencies could be reversed alone by the consumption of linoleic acid or linolenic acid and thus these fatty acids were heralded as essential fatty acids.

Essential fatty acid deficiency syndrome was uncommon in human adults and the first study in a human subject was done by the biochemist W. R. Brown, who himself went on a diet extremely low in fat for six months [50]. This experiment failed to develop any cutaneous or other visible abnormalities. With the appearance of parenteral nutrition based on a system of continuous fat-free infusion, patients developed alopecia (baldness due to hair failure), brittle nails, desquamating dermatitis, and increased susceptibility to infection.

The polyunsaturated fatty acids serve as cellular membrane phospholipid components which can influence the physicochemical characteristics of the lipid bilayer.

Changes in membrane lipids can modify the mobility and function of a variety of membrane proteins.

Linoleic acid is the most abundant PUFA in human skin. There is evidence that one functional role of linoleic acid is its involvement in the maintenance of the epidermal water barrier [51]. The physical structure of this water barrier is ascribed to sheets of stacked bilayers that fill the extracellular space of the uppermost layer of the epidermis. These lipid bilayers contain large amounts of linoleate rich sphingolipids.

The mechanism of the reversion of the symptoms of essential fatty acid deficiency (which include hyperproliferation and transepidermal water loss) by feeding with linoleic acid was elucidated in the last years at least for the symptom of hyperproliferation. In the epidermis linoleic acid is metabolized to 13-hydroxyoctadecadienoic acid (13-HODE) [52]. Studies showed that 13-HODE was incorporated into epidermal phosphatidylinositol 4,5-bisphosphate, resulting in the PLC catalyzed release of 13-HODE containing diacylglycerol [53]. It is thought that this molecule could modulate the activity of epidermal PKC and therefore influences epidermal hyperproliferation and differentiation.

The second important PUFA in the skin is arachidonic acid. It makes up to 9% of the total fatty acids in the epidermal phospholipids in human epidermis. It is the major metabolite of linoleic acid. Arachidonic acid is metabolized via the cyclooxygenase pathway, predominantly to the prostaglandins E2, F2a, and D2, and also via the 15-lipoxygenase pathway, predominantly to 15-hydroxyeicosatetraenoic acid (15-HETE) [54].

Prostaglandins play a central role in inflammation. 15-HETE was reported to improve the symptoms of psoriasis vulgaris after intralesional injection [55] and it was also reported to inhibit the 5-lipoxygenase activity and the generation of leukotriene B4 in neutrophils and basophils in vitro [56]. These *in vitro* effects suggest an anti-inflammatory potential.

1.4 PUFA and melanogenesis

From the work of Shono [57] at the beginning of the eighties of the last century it is well known that fatty acids have an influence on the activity of tyrosinase of B16 mouse melanoma cells *in vitro*. In the last ten years this effect was examined in further work. Altogether four papers were published about this topic by Ando and his co-workers.

It was shown that the unsaturated fatty acids oleic acid, linoleic acid and α-linolenic acid lead to a decrease in melanin content and tyrosinase activity in cultured mouse melanoma cells of the cell line B16-F10 [58]. The growth rate of the cells was not altered by the substances. In the same study it was shown that the fatty acids lighten ultraviolet-induced hyperpigmentation of the skin of guinea pigs. It was thought that this effect was caused by two distinct processes; first the inhibition of the production of melanin and second the accelerated turnover of the stratum corneum.

It was also shown that linoleic acid can activate isolated PKC from B16 cells *in vitro* [59].

Linoleic acid does not alter the tyrosinase mRNA level in B16 cells [60] and has neither inhibitory influence on the activity of isolated tyrosinase. In a recent study a possible mechanism for the regulatory effect of polyunsaturated fatty acids on melanogenesis of cultured mouse melanoma cells was proposed [61]. It is thought that in the presence of polyunsaturated fatty acids in the medium the proteolytic degradation of tyrosinase is enhanced.

1.6 Objectives for the thesis

As previously mentioned, the inhibiting effect of unsaturated fatty acids on melanogenesis is already well known. It is proven that the addition of oleic, linoleic or linolenic acid to the medium of cultures of B16 mouse melanoma cells leads to a decreased intracellular melanin content [58, 61]. In contrast addition of saturated fatty acids to the medium leads to an increase in intracellular melanin content. In both cases the melanin content correlates with the activity of tyrosinase, the main enzyme of melanin biosynthesis.

To date the inhibiting effect of unsaturated fatt acids on melanogenesis was shown in an *in vitro* mouse melanoma cell culture model and *in vivo* in an animal model (guinea pig). In the animal model it was shown that unsaturated fatty acids can lighten UV-induced tanning but it is not known if they have an influence on constitutive pigmentation, which is defined as the pigmentation which is genetically determined in the absence of stimulatory influences.

As cell culture model B16 mouse melanoma cells were used. From this cell line different sublines exist. The American cell bank ATCC lists 3 sublines that are named B16-F0, B16-F1 and B16-F10. The European cell line collection ECACC further lists a cell line named B16 melanoma 4A5. Therefore it seems that four sublines of the B16 line exist. These sublines are not well characterized and nothing is known about the differences between them, although B16 cells are often used in studies investigating melanin biosynthesis.

To date there exist neither *in vitro* nor *in vivo* studies of the influence of fatty acids on melanogenesis of normal human melanocytes. In first experiments with B16-F1 mouse melanoma cells and linoleic acid we were not able to detect an inhibition in tyrosinase activity.

Thus in this work the following questions should be answered:

What is the function of linoleic acid, linolenic acid (synonym α-linolenic acid), arachidonic acid and γ-linolenic acid on melanogenesis of B16-F10 cells and B16-F1 cells?

Is there a difference between the two sublines of the B16 mouse melanoma cell line in the reaction on the incubation with polyunsaturated fatty acids ?

Do polyunsaturated fatty acids also affect the melanin synthesis of cultured normal human melanocytes?

Which effects have polyunsaturated fatty acids in a pigmented 3D *in vitro* skin model?

2. Material and Methods

2.1. Cell culture

2.1.1 Isolation and cultivation of normal human melanocytes

Normal human melanocytes were isolated from human foreskins and cultured according to the method of Eisinger [62] with few modifications.

Culture media and solutions

PBS without Ca^{2+} and Mg^{2+} (PBSA)

137 mM	NaCl	[Merck 6404]	8.00 g/liter
2.68 mM	KCl	[Merck 4936]	0.20 g/liter
1.47 mM	KH_2PO_4	[Merck 4873]	0.20 g/liter
8.09 mM	$Na_2HPO_4 * 2H_2O$	[Merck 6580]	1.44 g/liter

PBS-EDTA

137 mM	NaCl	[Merck 6404]	8.00 g/liter
2.68 mM	KCl	[Merck 4936]	0.20 g/liter
1.47 mM	KH_2PO4	[Merck 4873]	0.20 g/liter
8.09 mM	$Na_2HPO_4 * 2H_2O$	[Merck 6580]	1.44 g/liter
0.537 mM	EDTA	[Sigma ED2SC]	0.20 g/liter

0.25 % Trypsin

2.5% Trypsin [Gibco25090-028] was diluted with PBSA.

Trypsin-EDTA

2.5% Trypsin [Gibco25090-028] was diluted with PBS-EDTA to a concentration of 0.05%.

Complete medium

	MEM with NEAA	[Gibco 41500-067]
100 units/ml	penicillin	[Gibco 15140-122]
0.1 mg/ml	streptomycin	[Gibco 15140-122]
0.25 μg/ml	Fungizone®	[Gibco 15290-018]

Washing medium

	MEM with NEAA	[Gibco 41500-067]
1000 units/ml	penicillin	[Gibco 15140-122]
1 mg/ml	streptomycin	[Gibco 15140-122]
2.5 μg/ml	Fungizone®	[Gibco 15290-018]

Growth medium

Complete medium without Fungizone® supplemented with:

5%	FBS	[Gibco 10091-148]
16 nM	PMA	[Sigma P 8139]
2.5 nM	cholera toxin	[Sigma C 8052]
0.1 mM	IBMX	[Sigma I 7018]

Method

Human foreskins from circumcisions were collected in complete medium and stored near the surgical area at 4°C. Specimens were delivered to the cell culture laboratory the same day. The skin was transferred to a laminar flow hood where the following preparations were carried out under aseptic conditions. The skin was immersed in washing medium for 5, 10 and 15 min, each time in fresh solution. This was followed by a wash with complete medium. After that the skin was transferred to a Petri dish epidermal side down, and using curved scissors, fat and connective tissue were removed. Then the skin was cut into small pieces of about 25 mm^2. The pieces were washed with PBSA, placed in 0.25% trypsin and kept at 4°C overnight (~16 h).

Following this incubation each piece of skin was removed from the trypsin solution, placed on a Petri dish (dermal side down) and held with forceps.
Using a second pair of forceps epidermis was peeled off and collected in trypsin/EDTA solution. A single cell solution was prepared by pipetting. The solution was transferred to a tube containing a small amount of FBS. The mixture was centrifuged for 10 min at 180 x g. The pellet was resuspended in growth medium, filtered through a 100 μm cell strainer [Falcon 2360] and the resulting cell suspension was seeded into cell culture dishes. Cells were cultured in a CO_2 incubator [Heraeus] at 37°C and 5% CO_2. Medium was changed 48 h later to remove unattached cells. Afterwards medium was changed twice a week and the cells were subcultured when confluence reached about 70%.

2.1.2 Elimination of fibroblast contamination

Contaminating fibroblasts were eliminated by differential trypsinization or by selective destruction of fibroblasts with geniticin.

a) Differential trypsinization

Medium was discarded and cells were washed with PBSA. After that 0.25% trypsin solution was added. The solution was removed after it had wet the surface of the cell culture dish. The cells were observed under the inverted microscope [Olympus]. Melanocytes detached faster from the surface than fibroblasts and were collected in growth medium and replated at a density of 1 x 10^4 cells/cm^2. If there were still contaminating fibroblasts, the procedure was repeated. Cells were cultured at the conditions described above and subcultured when confluence was reached.

b) Selective elimination with geneticin

If the method above did not succeed in eliminating the fibroblast contamination, the cells were selectively killed by treatment with geneticin. This method was described by Halaban et al. [63].
They used geneticin at a dose of 100 μg/ml to selectively kill fibroblasts in mixed cultures of melanocytes and fibroblasts. We treated contaminated cultures with a dose of 200 μg/ml geneticin for seven days as a dose of 100 μg/ml did not succeed in eliminating all fibroblasts. Melanocytes were not affected by this treatment.

2.1.3 Characterization of melanocytes

Melanocytes were identified by immuncytochemistry. Tyrosinase was specifically detected with an anti tyrosinase antibody that was visualized with a second antibody labeled with fluorescein.

Materials

- Cultures of normal human melanocytes
- Cultures of normal human fibroblasts
- Culture slides [Falcon]
- PBSA
- Growth medium for melanocytes
- Growth medium for fibroblasts:

	DMEM	[Gibco 31600-026]
10%	FBS	[Gibco 10091-148]
100 units/ml	penicillin	[Gibco 15140-122]
0.1 mg/ml	streptomycin	[Gibco 15140-122]

- Acetone [Merck]
- PBS-Tween:
  PBSA including 0.02% (w/v) Tween 20 [Bio-Rad 170-6531]

- Antibody buffer:
  PBSA including 1% (w/v) BSA [Sigma A-8551]
- Antibody I:
  Tyrosinase Ab-1 (T311) [NeoMarkers MS-800-P1]
- Antibody II:
  Goat anti mouse IgG fluorescein labeled [Pierce 31569]
- PolyDABCO:

0.3 g	Tris HCl	[Sigma T-3253]
2.2 g	Tris base	[Sigma T-1503]
20.0 ml	H_2O bidest.	
2.0 g	Polyvinyl alcohol	[Sigma P-8136]
4.5 ml	Glycerin	[Merck 4094]
0.75 g	DABCO	[Fluka 33480]

Tris HCl and Tris base were solubilized in 20 ml H_2O. Polyvinyl alcohol was added in portions to the solution under stirring. The solution was stirred for 30 min. Then glycerin was added and the solution was warmed until glycerin had dissolved and the solution became clear. Afterwards the solution was cooled down to RT and pH was set to 8.70 with 2 M HCl. The solution was stored at 4°C.

Method

The cells were cultured in culture slides in a humidified incubator at 37°C and 5% CO_2 for four days. The medium was discarded, the media chamber removed and the cells were washed with PBSA three times. After this the cells were fixed for 10 min with ice cold acetone on ice. After this fixation the slides were washed three times with PBS-Tween. Antibody I was diluted 1:100 with antibody buffer and 100 µl of the solution were pipetted on the culture slide. The culture slides were incubated for 45 min at RT in a humid chamber. After this incubation the slides were washed with PBS-Tween three times. Antibody II was diluted 1:50 with antibody buffer.

On each slide 100 µl of antibody solution were pipetted. The slides were incubated at RT in the dark in a humid chamber. After 45 min the slides were washed three times with PBSA and mounted with PolyDABCO. The specimens were examined under a fluorescence microscope [Olympus BX50] using an exciter filter BP460-490 [Olympus] and a barrier filter BA515IF [Olympus]. Photographs were taken using a reflex camera [Olympus OM-4 Ti] and 35 mm film [Kodak].

2.1.4 Cultivation of B16-F1 and B16-F10 mouse melanoma cells

B16-F10 (CRL-6475) and B16-F1 (CRL-6323) mouse melanoma cells were purchased from ATCC. These cell lines were isolated from the mouse strain C57BL/6J and deposited at ATCC by the Naval Biosciences Laboratory. The following cell line descriptions were given by ATCC:

B16-F1	Organism:	*Mus musculus* (mouse)
	Tissue:	melanoma
	Strain:	C57BL/6J
	Morphology:	fibroblast-like
	Depositors:	Naval Biosciences Laboratory
	Tumorigenic:	yes, in syngenic mice
	Note:	This cell line produces melanin which may cause the culture medium to turn brown/black. This is normal and should not be misinterpreted as contamination
B16-F10	Organism:	*Mus musculus* (mouse)
	Strain:	C57BL/6J
	Depositors:	Naval Biosciences Laboratory

The growth medium consisted of:

	DMEM	[Gibco 31600-026]
5%	FBS	[Gibco 10091-148]
100 units/ml	penicillin	[Gibco1 5140-122]
0.1 mg/ml	streptomycin	[Gibco1 5140-122]

The cells were grown in cell culture Petri dishes in a humidified CO_2 incubator at 37°C and 7% CO_2. Due to the rapid growth cells were subcultured twice a week on Monday and on Friday. Medium was changed on Wednesday. For subculturing the medium was discarded, the cells were washed with PBSA and little 0.25% trypsin solution was added. After it had wet the surface the solution was removed and the cells were incubated at 37°C for 5 min. After this incubation the cells had detached from the surface and were collected in culture medium. The cells were seeded at a density of ~1.7 x $10^3/cm^2$.

2.1.5 Determination of cell number

The cell number was determined using a cell counter CASY® 1 [Schärfe System]. For counting, monolayers had to be trypsinized and resuspended in medium.

Material

- Cell monolayer
- PBSA
- 0.25% Trypsin
- Growth medium
- CASY®ton [Schärfe System]
- Counting cups [Schärfe System]
- CASY® 1 [Schärfe System]

Method

The cell suspension was diluted 1:100 in 10 ml CASY®ton counting fluid in a disposable counting cup. The suspension was mixed well and the cell number was determined according to the manufacturer's instructions.

2.2 Test substances

The following substances were chosen to investigate the influence on melanogenesis of normal human melanocytes and mouse melanoma cells:

Linoleic acid	(all cis-9,12-Octadecadienoic acid)	[Sigma L-1012]
Arachidonic acid	(all cis-5,8,11,14-Eicosatetraenoic acid)	[Matreya 1042]
α-Linolenic acid	(all cis-9,12,15-Octadecatrienoic acid)	[Matreya 1024]
γ-Linolenic acid	(all cis-6,9,12-Octadecatrienoic acid)	[Matreya 1153]

The test substances were stored under nitrogen at –20°C to reduce oxidation of the unsaturated bonds.

Preparation of stock solutions

Stock solutions (50 mM) [Merck 1.00983] were prepared in ethanol and stored under nitrogen at –20°C.

2.3 Determination of cytotoxicity

Cytotoxicity of the test substances was determined using the neutral red uptake (NRU) method [64].

2.3.1 Cytotoxicity on normal human melanocytes

Material

- Free fatty acid free FBS:
  Free fatty acid free fetal bovine serum was prepared using three extractions with equal volumes of diethyl ether [Merck 1.00921] in a separating funnel, according to the method of Ando [58]. The residual ether was removed using an evaporator [Büchi].

- Test medium for NHM:

	MEM with NEAA	[Gibco 41500-067]
10%	free fatty acid free FBS	
100 units/ml	penicillin	[Gibco 15140-122]
0.1 mg/ml	streptomycin	[Gibco 15140-122]
16 nM	PMA	[Sigma P 8139]
0.1 mM	IBMX	[Sigma I 7018]

- NR-medium
  Test medium containing 50 μg/ml neutral red [Sigma N-4638]. NR-Medium was prepared 24 hours before use and incubated overnight at 37°C. Medium was centrifuged for 10 min at 1000 x g to spin down precipitated dye.
- NR-fixation solution
  1% (v/v) acetic acid [Merck 1.00063], 50% (v/v) ethanol [Merck 1.00983], 49% (v/v) H_2O bidest. The solution was stored at RT.
- Cultures of NHM
- 96 well cell culture plates [Sarstedt]
- Deep well plate [Eppendorf]
- Multipette® plus [Eppendorf]
- Multichannel pipette [Eppendorf]
- Test substances

Method

Cells were trypsinized and resuspended in test medium. The cell number was determined using the cell counter CASY® 1. The cell suspension was diluted to 2 x 10^5 cells/ml and using a multichannel pipette, 100 µl of the cell suspension were pipetted into each well of column 2 to 12 of a microtiterplate. Into column 1, 100 µl of test medium were added. This column served as a blank.
The microtiterplate was incubated in a humidified incubator at 5% CO_2 and 37°C.
After an incubation period of 24 h the medium was changed to medium containing various concentrations of test substance. In a deep well plate 1 ml of medium containing the following concentrations of test substance was prepared per well:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	CONTROL	50 µM LA	100 µM LA	150 µM LA	200 µM LA	250 µM LA	50 µM AA	100 µM AA	150µM AA	200 µM AA	250 µM AA
B												
C												
D												
E		0.5% EtOH	50 µM α-LA	100 µM α-LA	150 µM α-LA	200 µM α-LA	250µM α-LA	50 µM γ-LA	100 µM γ-LA	150 µM γ-LA	200 µM γ-LA	250 µM γ-LA
F												
G												
H												

The maximal concentration of solvent (ethanol) was 0.5%. The old medium was discarded and using a multichannel pipette 100 µl of the medium containing test substance were applied to the microtiterplate. The plate was incubated at 37°C and 5% CO_2 in a humidified incubator for 72 h.
After this incubation the medium was discarded and 100 µl NR-medium were pipetted into each well of the microtiterplate and the plate was incubated in the incubator for an additional hour.

Then the medium was discarded, the cells were washed with 100 µl/well PBSA and 100µl/well NR-fixation solution were added. The plate was incubated on a shaker for 15 min and the absorbance at 540 nm was determined using a microtiterplate reader.
Blank mean value was subtracted from readings and data were expressed as percent of untreated control. Mean values and standard deviations were calculated.

2.3.2 Cytotoxicity on B16 mouse melanoma cells

Material

- free fatty acid free FBS:
  Preparation see 2.3.1
- Test medium for mouse melanoma cells

	DMEM	[Gibco 31600-026]
10%	free fatty acid free FBS	
100 units/ml	penicillin	[Gibco1 5140-122]
0.1 mg/ml	streptomycin	[Gibco1 5140-122]

- NR-medium
  Preparation see 2.3.1
- NR-fixation solution
  Preparation see 2.3.1
- Cultures of B16-F1 and B16-F10 mouse melanoma cells
- 96 well cell culture plates [Sarstedt]
- Deep well plate [Eppendorf]
- Multipette® plus [Eppendorf]
- Multichannel pipette [Eppendorf]
- Test substances

Method

Cells were trypsinized and resuspended in test medium. The cell numbers of the suspensions were determined using the cell counter CASY® 1. The cell suspensions were diluted to 2.5 x 10^4 cells/ml and using a multichannel pipette, 100 µl of each cell suspension were pipetted into each well of column 2 to 12 of a 96 well cell culture microtiterplate. Into column 1, 100 µl of test medium were added. This column served as a blank. The microtiterplates were incubated in a humidified incubator at 5% CO_2 and 37°C.

After an incubation period of 24 h the medium was changed to medium containing various concentrations of test substance. In a deep well plate 1 ml of medium containing the following concentrations of test substance was prepared per well:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	CONTROL	31.25 µM LA	62.5 µM LA	125 µM LA	250 µM LA	500 µM LA	31.25 µM AA	62.5 µM AA	125 µM AA	250 µM AA	500 µM AA
B												
C												
D												
E		0.5% EtOH	31.25 µM α-LA	62.5 µM α-LA	125 µM α-LA	250 µM α-LA	500 µM α-LA	31.25 µM γ-LA	62.5 µM γ-LA	125 µM γ-LA	250 µM γ-LA	500 µM γ-LA
F												
G												
H												

The maximal concentration of solvent (ethanol) was 0.5%. The old medium was discarded and using a multichannel pipette, 100 µl of the medium containing test substance were applied to the microtiterplate. The plate was incubated at 37°C and 5% CO_2 in a humidified incubator for 72 h.

After this incubation the medium was discarded and 100 µl NR-medium were pipetted into each well of the microtiterplate. The plates were incubated in the incubator for an additional hour.

Then the medium was discarded, the cells were washed with 100 µl/well PBSA and 100 µl/well NR fixation solution were added. The plate was incubated on a shaker for 15 min and the absorbance at 540 nm was determined using a microtiterplate reader.
Blank mean value was subtracted from readings and data were expressed as percent untreated control. Mean values and standard deviations were calculated.

2.4 Cell culture and assay conditions

For the experiments different approaches were chosen for mouse melanoma cells and normal human melanocytes.

2.4.1 Mouse melanoma cells

2.4.1.1 Melanin content and tyrosinase activity

Cells from a running culture were detached from the culture vessel by trypsinization and collected in test medium for mouse melanoma cells (see 2.3.2). The cell number was determined and the following densities were seeded into 175 cm^2 flasks and 100 mm dishes:

4.5×10^5 cells/cm^2 (24 h incubation)
1.5×10^5 cells/cm^2 (48 h incubation)
8.5×10^4 cells/cm^2 (72 h incubation)

The volume of medium was 30 ml for the flasks and 10 ml for the dishes.
The flasks and dishes were incubated in a humidified atmosphere at 37°C and 5% CO_2 for three hours. After this incubation period almost all cells had settled to the surface and 0.5 % (v/v) of a 5 mM stock solution of the test substance in ethanol or of diluent alone were added to the medium.

The cells were further incubated in the CO_2 incubator for additional 24, 48 or 72 h. Each substance and each incubation time was tested in triplicate.
After the incubation time cells were harvested. The cells from the flasks were used for determination of melanin content (see 2.5.1) whereas the cells from the dishes served for the tyrosinase activity assay (see 2.6).

2.4.1.2 Determination of whole melanin

The assay conditions were the same as described in section 2.4.1.1 with the exception that 100 mm dishes were used. Medium volume for the dishes was 10 ml. At the end of the incubation times extracellular melanin content was determined according to the method described in section 2.5.3 and intracellular melanin was quantified as described in section 2.5.1.

2.4.1.3 Tyrosinase content

Western Blot

Cells were seeded at a density of 8.5 x 10^4 cells/cm^2 in 10 ml of test medium into 100 mm culture dishes. Three hours later the test substances were added to the medium as described in section 2.4.1.1. The dishes were cultured in the CO_2 incubator for an additional 72 h. Then the tyrosinase content was determined as described in section 2.8.

ELISA

Cells were seeded at a density of 2 x 10^4 cells/cm^2 in 2.5 ml of test medium into 6 well plates. Three hours later the test substances were added to the medium as described in section 2.4.1.1.

The plates were cultured in the CO_2 incubator for further 72 h. Then the tyrosinase content was determined as described (see 2.7).

2.4.1.4 Expression of tyrosinase gene

Cells were seeded at a density of 8.5 x 10^4 cells/cm^2 in 10 ml test medium into 100 mm culture dishes. 24 h after seeding, the medium was replaced by test medium containing the polyunsaturated fatty acids (25 μM; 0.5 % ethanol in medium) or an equal volume of diluent. 24 h later the cells were harvested, counted with the cell counter CASY® 1 [Schärfe System] and tyrosinase mRNA content was determined as described in section 2.9.

2.4.2 Normal human melanocytes

2.4.2.1 Melanin content

Cells were seeded at a density of 2.5 x 10^4 cells/cm^2 in 2.5 ml of test medium into 6 well plates. The test medium was the same as described before (see 2.3.1). The cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere. 24 h later, 100 μM of test substance or diluent (ethanol) alone were added. The concentration of diluent was 0.5% (v/v). The cells were incubated for additional 72 h. Then the cells were harvested and the melanin content was determined as described in section 2.5.3.

2.4.2.2 Tyrosinase activity

Cells were seeded at a density of 2.5 x 10^4 cells/cm^2 in 1 ml of test medium into 12 well plates. The test medium was the same as described before (see 2.3.1.). The cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere. After 24 h, 100 μM of test substance or diluent (ethanol) alone were added. The concentration of diluent was 0.5% (v/v).

The cells were incubated for additional 72 h. Then the cells were harvested and the tyrosinase activity was determined as described in section 2.6.2.

2.4.2.3 Tyrosinase content

Western Blot

Cells were seeded at a density of 1 x 10^4 cells/cm^2 in 10 ml of growth medium (see 2.1.1) into 100 mm dishes. The dishes were incubated at 37°C and 5% CO_2 in a humidified atmosphere. After 24 h the medium was changed and replaced with test medium containing 100 µM of test substance or diluent alone. The concentration of diluent was 0.5% (v/v). The dishes were incubated at 37°C and 5% CO_2 in a humidified atmosphere for further 72 h. Then the cells were harvested and the tyrosinase content was determined as described in section 2.8.

ELISA

Cells were seeded at a density of 1 x 10^4 cells/cm^2 in 30 ml of growth medium (see 2.1.1) into T150 flasks and cultured at 37°C and 5% CO_2 in a humidified atmosphere for two weeks.
Medium was changed twice a week. After this incubation period medium was changed and replaced by test medium containing 100 µM of test substance or diluent alone. The concentration of diluent was 0.5% (v/v). The cells were incubated for further 72 h. Then the cells were harvested and the tyrosinase content was determined as described in section 2.7.2.

2.4.2.4 Expression of tyrosinase gene

Cells were seeded at a density of 1 x 10^4 cells/cm^2 in 10 ml of growth medium (see 2.1.1) into 100 mm dishes. The dishes were incubated at 37°C and 5% CO_2 in a humidified atmosphere. After 24 h the medium was replaced by test medium containing 100 µM of test substance or diluent alone. The concentration of diluent was 0.5% (v/v). The dishes were incubated at 37°C and 5% CO_2 in a humidified atmosphere for further 24 h. Then the cells were harvested and the tyrosinase mRNA content was determined as described in section 2.9.

2.5 Determination of melanin content

The melanin content of mouse melanoma cells was determined using a method described by Ando [58]. For normal human melanocytes a method described by Nakajima [65] was used. Extracellular melanin was quantified using a method described by Bathnagar [66].

2.5.1 Method according to Ando

The cells were detached from the culture vessel by trypsinization. Approximately 10^7 mouse melanoma cells were pelleted by centrifugation at 1000 x g for 5 min. The pellet was washed twice with PBSA. After a further centrifugation step the supernatant was decanted and the pellet resuspended in 200 µl of WFI. By addition of 1 ml ethanol/ether (1:1) opaque substances, other than melanin, were removed. The mixture was incubated at RT for 15 min and then centrifuged at 3000 x g for 5 min. The pellet was suspended in 1 ml of 1M NaOH containing 10% (v/v) DMSO. The mixture was incubated on a thermomixer at 80°C and 1000 rpm for 30 min.

The absorbance was measured at 450 nm and, using a standard curve of synthetic melanin in 1 M NaOH containing 10% (v/v) DMSO, melanin content of the sample and melanin content per cell was calculated.

2.5.2 Method according to Nakajima

In this assay cells were blotted on a nitrocellulose membrane [1.2 μm pore size; Sartorius] using a Bio-Dot microfiltration apparatus [Bio-Rad]. The cells were detached from the culture vessel by trypsinization and collected in PBSA containing 1 mg/ml trypsin inhibitor [Invitrogen]. The cell number was determined using the cell counter CASY® 1 [Schärfe System] and approximately 2 x 10^5 cells were blotted on a membrane and fixed by air drying. An image was taken from the membrane using the Camag Reprostar 3 cabinet [Camag], a digital camera [Hitachi] and the software VideoStore [Camag]. The picture was transformed into gray scale and the density of cell blots was analyzed with Scion Image software [Scion Corporation]. Data in each experiment were normalized by the control value and expressed as percentage of the control.

2.5.3 Method according to Bathnagar

With this assay the melanin secreted to the medium by mouse melanoma cells was estimated.
At the end of the incubation with test substances the medium was decanted and centrifuged at 800 x g for 10 min to remove floating cells. The absorbance of the centrifuged medium was measured at 400 nm. Using a standard curve of synthetic melanin in medium melanin content of the sample and melanin content per cell was calculated.

2.6 Determination of tyrosinase activity

The tyrosinase activity was determined using the method described by Ando [58] and a second one described by Winder and Harries [67]. The method of Ando was used only for B16 mouse melanoma cells whereas with the assay of Winder and Harris (MBTH-method) the tyrosinase activity of NHM and mouse melanoma cells was determined.

2.6.1 Method according to Ando

After treatment with test substances the cells were detached from the surface of the tissue culture vessel by trypsinization. The cells were resuspended in 10 ml PBSA containing 5% FBS and the cell number was determined using the cell counter CASY® 1. About 2.5 x 10^6 cells were pelleted by centrifugation at 1000 x g for 5 min. The pellet was washed twice with 1 ml of PBSA. Then the cells were lysed on ice for 15 min in 1 ml of 0.5% sodium deoxycholate [Sigma] in WFI. The cell extract was cleared by centrifugation at 5000 x g for 10 min. In a cuvette 250 µl of the cleared cell extract was mixed with 750 µl of 0.1% L-DOPA in 0.1M phosphate buffer (pH 6.8). The increase of absorbance at 475 nm over a period of 10 min at 37°C was measured using a spectrophotometer [Pharmacia] and a heat insert.

2.6.2 MBTH-method

The original assay described by Winder and Harris was modified with the purpose to measure the tyrosinase activity in a microtiterplate format. About 3.0 x 10^5 cells were pelleted by centrifugation at 1000 x g for 5 min. The pellet was washed twice with 1 ml of PBSA and lysed in 500 µl of lysis buffer for 30 min at 8°C and 750 rpm on a shaker. Lysis buffer consisted of 1% (v/v) Triton X-100 [Sigma] in PBSA. The cell extract was cleared by centrifugation at 10'000 x g for 5 min.

In a microtiterplate the following volumes were pipetted per well:

95 µl	MBTH-buffer	(100 mM sodium phosphate pH 7.1, 4% by vol. N,N-dimethylformamide)
80 µl	cell extract	
50 µl	MBTH solution	(20.7 mM MBTH in WFI)
25 µl	L-DOPA solution	(5 mM L-DOPA in PBSA)

Samples were measured in triplicate. The increase of absorbance at 492 nm at RT over a period of 20 min was measured using a microtiterplate reader [Titertek].

2.7 Quantitative analysis of tyrosinase content of cell lysates by ELISA

The tyrosinase content of crude cell extracts of B16 mouse melanoma cells and NHM was determined using an ELISA. As there exists no commercially available test kit, an assay was developed according the method described by Crowther [68].

Assay principle

The assay is a competitive indirect assay. Competition assays are defined as assays where the detecting antibodies are added with the sample simultaneously. The first step in this principle is to coat the surface with the antigen. In our case this is the control peptide. This peptide is used as there is no human or mouse tyrosinase commercially available. Unbound antigen is washed away. While coating the plate with antigen, samples or a dilution range of antigen (control peptide) are incubated with a fixed amount of antibody (anti-tyrosinase) in a separate microtiterplate. These solutions are transferred to the coated plate, where antibodies that are not blocked by antigen bind to the antigen adsorbed on the plastic surface. Unbound antibodies are washed away. Bound antibodies are detected with an enzyme labeled anti-species antibody that binds specifically to the first antibody.

Unbound anti-species antibody is washed away and bound anti-species antibody is detected and quantified by addition of a specific substrate for the enzyme.

2.7.1 Quantification of mouse tyrosinase

2.7.1.1 Solutions and buffers

- Goat anti-tyrosinase antibody (mouse)
- Goat anti-tyrosinase IgG [RDI RDI-RTTYROSINabG]
  200 μg in 0.5 ml PBS with 100 μg BSA and 0.1% NaN_3. Store at 4°C.
- Control peptide for goat anti-tyrosinase antibody (mouse)
  This peptide is about 20 amino acids in size and corresponds to the C-terminus of mouse tyrosinase. The peptide was used to generate the antibody described above.
  100 μg in 0.5 ml PBS with 100 μg BSA and 0.1% NaN_3. Store at 4°C.
- Rabbit anti-goat IgG labeled with alkaline phosphatase
  Rabbit anti-goat IgG AP labeled [Pierce 31300]
  0.6 mg/ml in 0.01 M Tris HCl, 0.25 M NaCl, pH 8.0, 0.05% NaN_3, 15 mg/ml BSA. Store at 4°C.
- Goat IgG
  Goat IgG purified technical grade [Sigma I 9140]
- Tris buffered saline (TBS)
  TBS 10 x concentrated [Bio-Rad 170-6435] was diluted with WFI [Pentapharm]. The buffer was stored at 4°C.
- Wash buffer/Blocking solution

	TBS	[Bio-Rad 170-6435]
1% (w/v)	BSA	[Sigma A-8551]
0.05% (v/v)	Tween 20	[Bio-Rad 170-6531]

  The solution was stored at 4°C.

- Carbonate/Bicarbonate buffer

  13 ml 0.2M $Na_2CO_3*10H_2O$ [Merck 6384]

  37 ml 0.2M $NaHCO_3$ [Merck 1.06329]

  mix and dilute with WFI to 200 ml. Store at 4°C.

- Diethanolamine buffer

  10 % (w/v) Diethanolamine [Sigma D-0681]

  0.5mM $MgCl_2*6H_2O$ [Merck 5833]

  set pH at 9.8 with 5 M HCl. Store buffer at 4°C.

- Color solution

  1 mg/ml p-Nitrophenylphosphat [Sigma 104-105] was solubilized in diethanolamine buffer. The solution was always prepared fresh before use.

2.7.1.2 Determination of optimum of conjugate concentration

Optimal concentration of conjugate was determined using chessboard titration method.

Material

- Nunc-Immuno™ plate [Nunc]
- Rabbit anti Goat IgG with AP
- Goat IgG solution
- Carbonate/bicarbonate buffer
- Wash buffer
- Color solution
- Multichannel pipettes [Eppendorf]

Method

Using the multichannel pipette, 50 µl of carbonate/bicarbonate buffer were pipetted to each well of a Nunc-Immuno™ plate.

The antigen (Goat IgG solution) was diluted to 10 μg/ml in carbonate/bicarbonate buffer and 50 μl of the diluted antigen solution were pipetted into each well of column 1. With the multichannel pipette the solution was mixed and 50 μl were transferred to column 2, mixed and 50 μl transferred to column 3, and so on, to column 11. No antigen was pipetted to column 12. The plate was sealed with a foil [Sarstedt] and then incubated on a shaker at RT for 2 h.
Then the solution was discarded and the wells were washed four times with 100 μl of wash buffer. To each well of the microtiterplate 50 μl of wash buffer were added. The conjugate (Rabbit anti goat labeled with AP) was diluted 1:1000 with wash buffer and 50 μl of this solution were pipetted into row A. The solution was mixed and 50 μl were transferred to row B, mixed, 50 μl transferred to row C, and so on, to row H. The plate was sealed with a foil and incubated on a shaker at RT for 1 h.
Then the solution was discarded, the plate washed as described above and to each well 100 μl of color solution were pipetted. The plate was incubated at RT for 15 min. The absorbance was measured at 405 nm with a microplate reader [Titertek]. The data were transferred to EXCEL and Titration curves were created for each row with antigen dilution on x-axis and optical density on y-axis.

2.7.1.3 Determination of optimum concentration of control peptide and anti tyrosinase antibody

Optimal concentrations of the control peptide and anti tyrosinase antibody were determined using chessboard titration method.

Material

- Nunc-Immuno™ plate [Nunc]
- Goat anti-tyrosinase antibody
- Control peptide for goat anti-tyrosinase antibody
- Rabbit anti Goat IgG with AP
- Carbonate/bicarbonate buffer
- Wash buffer
- Color solution
- Multichannel pipettes

Method

The procedure was the same as described before (2.4.1.2). In a first step the control peptide at a concentration of 20 μg/ml was diluted from column 1 to column 11. The plate was incubated at RT on a shaker for 2 h. Then the plate was washed four times and the anti tyrosinase antibody at a concentration of 20 μg/ml was diluted in wash buffer from row A to row H. The plate was incubated on a shaker for 1 h at RT. The plate was washed four times and then 50 μl of conjugate, diluted 1:8000 in wash buffer, were added to each well. An incubation step on a shaker at RT for 1 h followed. After this incubation the plate was washed four times and 100 μl of color solution were added to each well. After a color development for 15 min the absorbance was measured at 405 nm. Data were transferred to EXCEL and Titration curves were created for each row and each column with dilution of control peptide, resp. anti tyrosinase antibody, on x-axis and optical density on y-axis.

2.7.1.4 Specificity of the ELISA

To check the specificity of the ELISA, crude cell extracts of four cell lines were analyzed with the assay. Two cell lines were of mouse melanoma origin, one was a human keratinocyte cell line and one a mouse fibroblast cell line.

Cell culture and preparation of cell extracts

Material

- Cultures of B16-F1 and B16-F10 mouse melanoma cells
- Cultures of HaCaT human keratinocyte cell line
- Cultures of 3T3 mouse fibroblast cell line
- Culture dishes
- Incubator
- PBSA
- TBS
- Culture medium consisted of DMEM and 5% FBS and antibiotics for all four cell lines
- Lysis buffer
  1% Triton X-100 [Sigma T-8787] in WFI with protease inhibitor cocktail [Roche 1836170]

The cells were cultured in ∅ 100 mm culture dishes at 37°C and 7% CO_2. At confluence the cells were trypsinized and the cell number was determined with the cell counter CASY® 1. The cells were pelleted by centrifugation at 200 x g for 5 min. After washing the pellets twice with TBS, they were solubilized in 100 µl/1.5 x 10^6 cells of lysis buffer. The suspensions were incubated on a shaker at 4°C at 1000 rpm for 30 min. Then the cell extracts were cleared by centrifugation at 2000 rpm at 4°C for 10 min.

The supernatants were transferred into new tubes and centrifuged at 13'000 rpm at 4°C for 30 min. From the supernatants protein and tyrosinase contents were determined.

Determination of tyrosinase content by ELISA

Material

- Nunc-Immuno™ plate [Nunc]
- Microtiterplate [Sarstedt]
- Goat anti-tyrosinase antibody
- Control peptide for goat anti-tyrosinase antibody
- Rabbit anti goat IgG with AP
- Carbonate/bicarbonate buffer
- Wash buffer
- Color solution
- Multichannel pipettes

Method

To a Nunc-Immuno™ plate 50 µl of antigen (control peptide) at a concentration of 0.5 µg/ml in carbonate/bicarbonat buffer were applied. The binding of the antigen to the wells was performed at RT for 2 h under shaking. At the same time, 50 µl of known amounts of antigen as standard, or unknown amounts in samples, were incubated with 50 µl of 1:200 diluted goat anti-tyrosinase antibody in a microtiterplate at RT for 2 h under shaking.
After this incubation the solution from the Nunc-Immuno™ plate was discarded and the wells were washed four times with 100 µl TBS. The preincubated mixture of antigen and antibody was transferred to the coated plate and incubated at RT under shaking for 1 h.

Then the plate was washed four times with TBS and 50 µl of rabbit anti-goat antibody labeled with AP (1:4000 in wash buffer) were given to each well. After an incubation step at RT for 1 h under shaking, the plate was washed four times and 100 µl of color solution were pipetted into each well. The color development was measured after 15 min at 405 nm with a microtiterplate reader.
Data were transferred to EXCEL and a standard curve was created. With the help of this standard curve the tyrosinase concentrations of the samples were calculated.

2.7.1.5 ELISA for the quantification of mouse tyrosinase

Material

- Samples
- Nunc-Immuno™ plate [Nunc]
- Microtiterplate [Sarstedt]
- Goat anti-tyrosinase antibody
- Control peptide for goat anti-tyrosinase antibody
- Rabbit anti goat IgG with AP
- Carbonate/bicarbonate buffer
- Wash buffer
- Color solution
- Multichannel pipettes

Method

Control peptide was diluted with carbonate/bicarbonate buffer to a concentration of 0.5 µg/ml and 50 µl of this solution were applied to a Nunc-Immuno™ plate. The plate was sealed with foil and incubated at RT under shaking for 2 h.

At the same time the samples and the standards were incubated with a known amount of goat anti-tyrosinase antibody in a microtiterplate. For this purpose 60 µl of wash buffer were given to each well of the microtiterplate. An additional 30 µl of wash buffer were given to the wells 3 to 12 in row A and E.

To well A1 and B1 60 µl of control peptide at a concentration of 0.63 µg/ml in wash buffer were pipetted. Into the wells containing 90 µl of wash buffer (A3 to 12 and E3 to 12) 30 µl of sample were given. With a multichannel pipette a serial dilution with factor 2 was made from row A to D and from E to H for the samples and a serial dilution with factor 2 from row A to H for the standards. The plate was sealed with foil and incubated at RT for 2 h under shaking.

Then the solution from the Nunc-Immuno™ plate was discarded, the wells were washed four times with 100 µl of TBS and 100 µl of the preincubated samples and standards were transferred to the corresponding wells of the coated plate. An incubation step of 1 h at RT under shaking followed. After this incubation the plate was washed four times and 100 µl of conjugate at a dilution of 1:4000 in wash buffer were given to each well. After incubation at RT for 1 h under shaking the plate was washed four times and 100 µl of color solution were added to each well. After a color development of 15 min the absorbance at 405 nm was measured with a microtiterplate reader. Data were transferred to EXCEL and a standard curve was created. Using this standard curve the concentrations of the samples were calculated. Tyrosinase content was expressed as nanogramm tyrosinase per milligramm protein.

2.7.2 Quantification of human tyrosinase

For the quantification of human tyrosinase no special experiments were done for determination of optimum concentrations of reagents.

The assay design and the concentrations were the same as for mouse tyrosinase. The only exception was the slight modification of the concentrations for the standard curve.

2.7.2.1 Solutions and buffers

- Goat anti-tyrosinase antibody (human)
  Goat anti-tyrosinase IgG [RDI-TYROSINabG]
  200 μg in 1 ml PBS with 0.2% gelatin and 0.1% NaN_3. Store at 4°C
- Control peptide for goat anti-tyrosinase antibody (human)
  This peptide is about 20 amino acids in size and corresponds to the C-terminus of human tyrosinase. The peptide was used to generate the antibody described above.
  100 μg in 0.5 ml PBS with 100 μg BSA and 0.1% NaN_3. Store at 4°c
- Rabbit anti-goat IgG labeled with alkaline phosphatase
  (see 2.7.1.1)
- Tris buffered saline (TBS)
  (see 2.7.1.1)
- Wash buffer/Blocking solution
  (s. 2.7.1.1)
- Carbonate/bicarbonate buffer
  (s. 2.7.1.1)
- Diethanolamine buffer
  (s. 2.7.1.1)
- Color solution
  (s. 2.7.1.1)

2.7.2.2 Specificity of the ELISA

The specifity of the ELISA was checked by analyzing cell extracts from NHM and 3T3 mouse fibroblasts with this assay.
The experiment was done as described in section 2.7.1.4.

2.7.2.3 ELISA for the quantification of human tyrosinase

Assay was done as described before (2.7.1.5).

2.8 Quantitative analysis of tyrosinase content of cell lysates by Western Blot

The Western Blot method was used as a second method to determine the content of tyrosinase of B16 mouse melanoma cells and NHM. To do this, cells were solubilized and the cell extracts were cleared by centrifugation. The mixture of proteins in these extracts was separated by SDS-PAGE and the proteins were blotted on PVDF membranes. Tyrosinase and GAPDH were specifically detected with antibodies. GAPDH served as a loading control.

2.8.1 SDS-PAGE

Material

- Cell lysis
  Cell lysis was performed as described before (2.7.1.4) using the following lysis buffer:
  1% Nonidet P40 [Sigma N-3516], 0.01% SDS [Merck 1.13760] in 0.1 M Tris HCl (pH 7.2) with protease inhibitor cocktail [Roche 1836170]
- Running buffer
  Tris/Glycine/SDS buffer [BioRad 161-0732]
  25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3

- Sample buffer
  Laemmli sample buffer [BioRad 161-0737] with 5% (v/v) β-Mercaptoethanol [Sigma M-7522]
- Gel
  4-15% Tris-HCl Ready Gel [BioRad 161-1122]
- Marker
  Precision Protein Standards [BioRad 161-0372]
- Mini-Protean II electrophoresis cell [BioRad 165-2940]
- PowerPac 300 [BioRad 165-5051]
- Thermomixer Comfort [Eppendorf]
- Centrifuge 5410 [Eppendorf]

Method

Samples were mixed with the same amount of sample buffer and incubated at 99°C for 5 min. After this denaturation the samples were cooled down to RT and centrifuged for 2 min at 500 x g.
The electrophoresis chamber was prepared according to the manufacturer's instructions.
10 µg of sample and 10 µl of marker were separated at 200 V, V=const. and 60 mA per gel for 45 min.

2.8.2 Blotting

Material

- Filter paper [BioRad 170-3966]
- PVDF Membrane (0.2 µm) [BioRad 162-0174]
- Transfer buffer
  Tris/Glycine buffer [BioRad 161-074]
  25 mM Tris, 192 mM Glycine, 20% (v/v) Methanol [Merck], pH 8.3

- Tris buffered saline (TBS) [BioRad 170-6435]
  20 mM Tris, 500 mM sodium chloride, pH 7.5
- TBS with Tween (TTBS)
  20 mM Tris, 500 mM sodium chloride, 0.05% Tween-20, pH 7.5
- Blocking buffer
  TBS with non fat dry milk [BioRad 170-6404]
  20 mM Tris, 500 mM sodium chloride, 5% (w/v) non fat dry milk, pH 7.5
- Antibody
  Goat anti-tyrosinase (mouse) IgG [RDI RDI-RTTYROSINabG]
  200 μg in 0.5 ml PBS with 100 μg BSA and 0.1% NaN_3. Store at 4°C

  Goat anti-tyrosinase (human) IgG [RDI RDI-TYROSINabG]
  200 μg in 0.5 ml PBS with 100 μg BSA and 0.1% NaN_3. Store at 4°C

  Mouse anti-GAPDH (rabbit) IgG [RDI RDI-TRK5G4-6C5]
  4.16 mg/ml in PBS with 0.1% NaN_3, pH 7.4

  Goat anti-mouse IgG AP-Conjugate [BioRad 170-6520]

  Rabbit anti-goat IgG AP-Conjugate [Pierce 31300]
- AP color development solution
  AP conjugate substrate kit [BioRad 170-6432]
  The solution was prepared freshly before use according to the manufacturer's instructions.

Method

During protein electrophoresis the PVDF membrane and the filter paper sheets were equilibrated in transfer buffer at 4°C for 45 min.
After the cell lysates were separated by SDS-PAGE the gel was equilibrated in transfer buffer for 15 min.
The proteins from the gel were blotted on the membrane according to the manufacturer's instructions, using the semidry blotting apparatus.

The blotting conditions were 10 V, V=const., 220 mA for 45 min.
Then the gel was separated from the membrane, rinsed with TBS and stained with GelCode Blue reagent [Pierce 24590] to check the transfer.
The membrane was rinsed with TBS and blocked in blocking buffer for 30 min. Then it was rinsed with TTBS and incubated in antibody buffer containing goat anti tyrosinase antibody diluted 1:200 and mouse anti rabbit GAPDH antibody diluted 1:4200. After an incubation of 1 h at RT, the membrane was rinsed with TTBS and incubated with rabbit anti goat antibody labeled with AP diluted 1:3000 in antibody buffer for 30 min. After washing the membrane with TTBS, it was incubated for 30 min with goat anti mouse IgG labeled with AP diluted 1:3000 in antibody buffer.
The membrane was washed with TTBS and TBS and placed in AP color development solution. After 15 min bands appeared and color development was stopped by rinsing the membrane with tap water.
The membrane was dried, photographed and the bands were densitometrically analyzed using the software Scion Image for Windows Beta 4.0.2 [Scion Corporation].

2.9 Tyrosinase gene expression

Tyrosinase gene expression was determined by quantification of tyrosinase mRNA levels. The tyrosinase mRNA levels were measured by quantitative real-time TaqMan® PCR.
The TaqMan® RT-PCR method uses the endogenous 5` nuclease activity of AmpliTaq Gold® DNA polymerase to digest an internal oligonucleotide probe labeled with both a fluorescent reporter dye and a quencher [69]. Assay results are detected by measuring changes in fluorescence that occur during the amplification cycle. The fluorogenic probe is digested, uncoupling the dye and quencher labels and causing an increase in the fluorescence signal that is proportional to the amplification of target DNA.

2.9.1 Isolation of total RNA from cells

Total RNA was isolated using the „High Pure RNA Isolation Kit" [Roche 1 828 665] following the manufacturer's instructions. The isolated RNA was quantified spectrophotometrically at 260 nm using UV-transparent plastic cuvettes [Eppendorf 0300 106.300] and an Ultrospec III spectrophotometer [Pharmacia]. RNA content was calculated using the following formula:

$$Concentration\,of\ \ RNA\ sample\,(\mu g/ml) = 40 \times OD_{260nm} \times dilution\ \ factor$$

RNA samples were stored at –20°C.
The integrity and purity of the isolated RNA were checked by denaturating agarose gel electrophoresis and ethidium bromide staining.

Material

- RNase-free water
  0.1% (v/v) DEPC [Sigma] in WFI. The solution was shaken vigorously to bring the DEPC into solution and stored at 37°C for 12 h. Then it was autoclaved for 15 min at 121°C to remove any trace of DEPC.
- 10 x FA Gel buffer
  200 mM 3-(N-morpholino) propanesulfonic acid (MOPS)
  50 mM sodium acetate
  10 mM EDTA
  adjust pH to 7.0 with 1 M NaOH
- 1 x FA Gel Running buffer
  100 ml 10 x FA gel buffer
  20 ml 37% formaldehyde
  880 ml RNase-free water

- 5 x RNA Loading buffer

16 µl	saturated aqueous bromophenol blue solution
80 µl	500 mM EDTA (pH 8.0)
720 µl	37% formaldehyde
3084 µl	formamide
4 ml	10 x FA gel buffer

add RNase-free water to 10 ml.

Method

- 1.2% FA gel preparation

1.2 g	agarose
10 ml	10 x FA gel buffer

add RNase-free water to 100 ml.

The mixture was heated in the microwave to melt the agarose. The solution was cooled down to 65°C and 1.8 ml of 37% formaldehyde and 1 µl of a 10mg/ml ethidium bromide stock solution was added. The mixture was poured onto the gel casting tray.

- RNA sample preparation

1 volume of 5 X loading buffer was mixed with 4 volumes of RNA sample. The solution was mixed well, incubated at 65°C for 5 min and chilled on ice.

- Gel running conditions

The gel was run at 100 V, V=const. for 1 h.

Bands of separated RNA were detected using an UV lamp [Camag] at 366 nm.

2.9.2 Synthesis of cDNA

mRNA was specifically reverse transcribed from total RNA into single stranded cDNA using an Oligo-p$(dT)_{15}$ Primer and the „1st Strand cDNA Synthesis Kit for RT-PCR" [Roche].
The cDNA synthesis reactions were carried out according to the manufacturer's instructions.
cDNA samples were stored at –20°C.

2.9.3 Quantitative real-time TaqMan® PCR

To standardize the amount of cDNA added to the PCR reaction, the amplification of the mouse gene GAPDH was used as an endogenous control. For amplification and quantitation of GAPDH the primers and the TaqMan® probe were designed using the software Primer Express™ [Applied Biosystems] and the following DNA sequences:

- Mouse GAPDH (EMBL, M32599)
- For human GAPDH a commercially available kit was used [Applied Biosystems 4310884E]

For the amplification and quantitation of tyrosinase cDNA primers and TaqMan® probe construction based on the following published DNA sequence:

- Mouse tyrosinase (EMBL, M24560)
- Human tyrosinase (EMBL, M27160)

The primer nucleotide sequences are in detail:

Mouse
Forward primer 5‘-CGA CCT CTT TGT ATG GAT GCA TTA-3‘
Reverse primer 5‘-CCC AGT TAG TTC TCG AAT TTC TTG T-3‘
Probe 5‘-VIC-ACA TTG ATT TTG CCC ATG AAG CAC CAG-TAMRA-3‘.

Human
Forward primer 5‘-TCA ATG GAT GCA CTG CTT GG-3‘
Reverse primer 5‘-ACC GCA ACA AGA AGA GTC TAT GC-3‘
Probe 5‘-VIC-ACA TTG ATT TTG CCC ATG AAG CAC CAG-TAMRA-3‘.

To ensure that the primers were specific for tyrosinase sequence, alignments were performed based on the following published DNA sequences for tyrosinase related protein 1 (TRP1) and tyrosinase related protein 2 (TRP2):

Mouse
TRP1 (EMBL, X03687)
TRP2 (EMBL, X63349)

Human
TRP1 (EMBL, X51420)
TRP2 (EMBL, D28767)

All primers were synthesized by Genset, France. Probes were synthesized by Applied Biosystems, Germany.

PCR conditions were:

Mouse

25 µl TaqMan Universal PCR Master Mix [Applied Biosystems]
2 µl Forward primer (8 µM)
2 µl Reverse primer (8 µM),
1 µl Probe (8 µM)
5 µl cDNA sample
15 µl H_2O

Human

25 µl TaqMan Universal PCR Master Mix [Applied Biosystems]
2 µl Forward primer (8 µM)
2 µl Reverse primer (8 µM),
1 µl Probe (8 µM)
10 µl cDNA sample
10 µl H_2O

After a hot start 45 cycles were performed as follows: 15 s at 95°C, 1 min at 60°C.
The relative quantitation was carried out using the so-called „comparative C_T method"[70].
Student's t-Test, ANOVA and Dunnett test were used for the statistical analysis of the results.

2.10. Influence of PUFA on melanogenesis of MelanoDerm™

MelanoDerm™ is a pigmented skin model produced by Mattek that consists of normal, human keratinocytes and melanocytes which have been cultured together to form a multilayered, highly differentiated model of the human epidermis. The tissues are grown on cell culture inserts at the air-liquid interface allowing the topical application of test substances. The serum free medium contains no artificial stimulators of melanogenesis such as TPA and IBMX.

Culture of the tissues and application of test substance

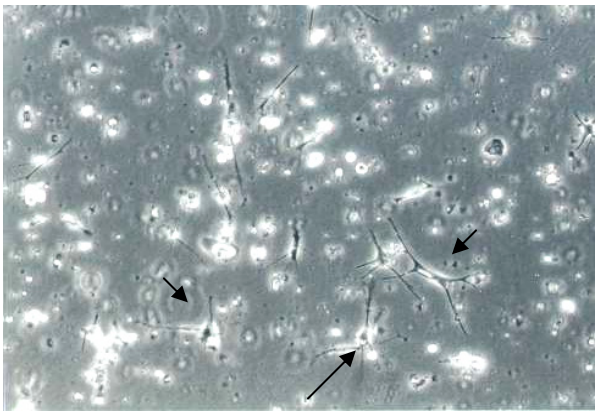
At the day of arrival the tissues were equilibrated and cultivated according to the manufacturer's instructions for 24 h. Then medium was changed and 25 µl of 1% (v/v) test substance in 10% ethanol (v/v) in WFI, 25 µl of diluent or nothing was topically applied. The tissues were incubated for 21 days. Every second day medium was changed and new test substance or diluent was applied.
In the second experiment 25 µl of 2.5% (v/v) test substance in 25% ethanol or DMSO (v/v) in WFI, 25 µl of diluent alone or nothing was applied. The tissues were cultivated for three weeks and every second day medium was changed and new test substance or diluent was applied.
At the end of the treatment the tissues were washed with PBSA and microscopic and macroscopic images were taken. Using the image analysis software Scion Image [Scion Corporation] the level of darkening was determined.
Melanin content in the tissues was determined by degradation of the tissues with proteinase K and extraction of melanin according to the manufacturer's instructions. Data were normalized to the control value and expressed as percentage of the control ± standard deviation.

3. Results

3.1.Cell culture

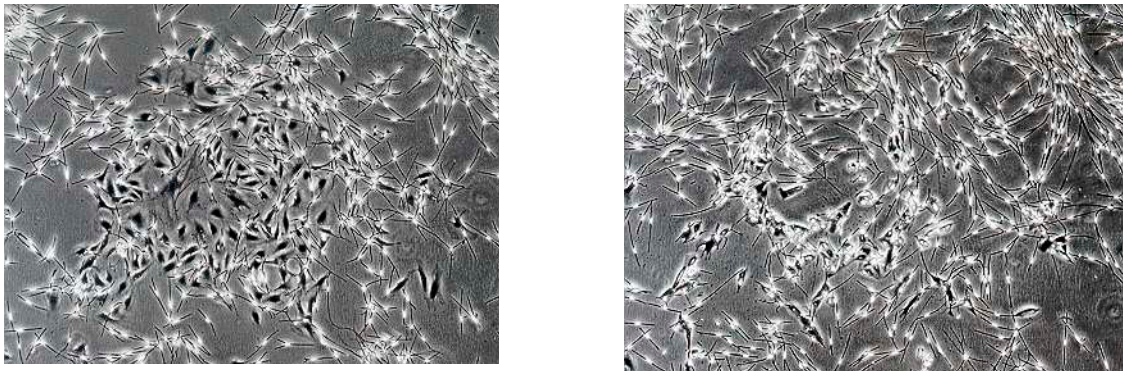
3.1.1 Normal human melanocytes

When single cell suspensions obtained from epidermis by trypsin treatment were plated in the presence of PMA and IBMX, predominantly melanocytes attached and grew. Attached and spred melanocytes were clearly visible within 24-48 h after seeding (see **picture 1**).



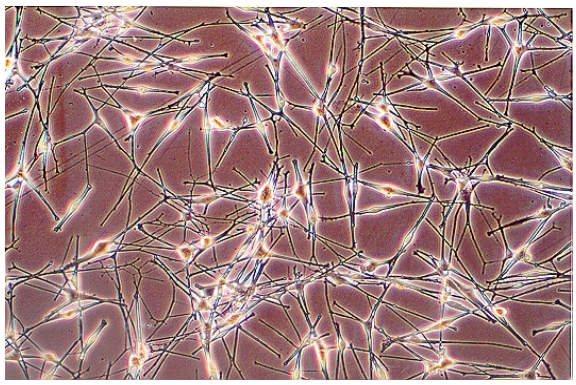
Picture 1 Melanocyte primary culture 48 hours after seeding. Arrows indicate attached melanocytes. (60x)

PMA did not interfere with the growth of fibroblasts and IBMX only partially supressed their growth. Replacement of IBMX with 10 nM of cholera toxin did neither succeed in suppressing fibroblast growth. Separation of melanocytes from fibroblasts by differential trypsination was successful in many cases but if fibroblast contamination could not be stopped by this method, treatment of the cultures with the antibiotic geneticin selectively eliminated fibroblasts within one week without killing melanocytes (see **picture 2**).



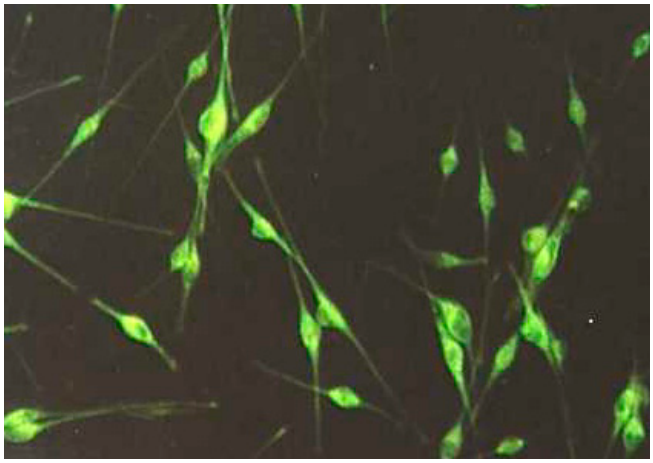
Picture 2 Melanocyte culture with contaminating fibroblasts (left).
The same culture after treatment with 200 μg/ml geneticin for seven days (right).

Melanocytes showed a spindle shaped bi- or tripolar morphology (see **picture 3**) and a slow growth with a twofold increase in 30 days.



Picture 3 Melanocyte culture at passage five (150x)

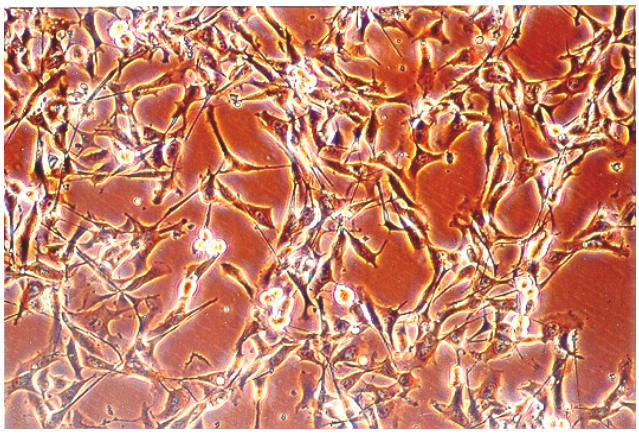
Melanocytes were identified by their morphology and immuncytochemistry using a specific monoclonal antibody against human tyrosinase.
Human melanocytes in culture were bi- or multiplar dendritic with a bright shining cell body.
Stained cells showed a bright fluorescence over the whole cell body (see **picture 4**) while fibroblasts showed no fluorescence (picture not shown).



Picture 4 Immunfluorescence labelling of tyrosinase in cultured normal human melanocytes (200x)

3.1.2 Mouse melanoma cells

B16-F1 and B16-F10 mouse melanoma cells showed a fibroblast-like morphology in culture (see **picture 5**).



Picture 5 Culture of B16 mouse melanoma cell line (200x)

Both cell lines showed a fast growth with a population doubling time that was dependent of seeding density (see **Fig. 3.1** and **Tab. 3.1**).

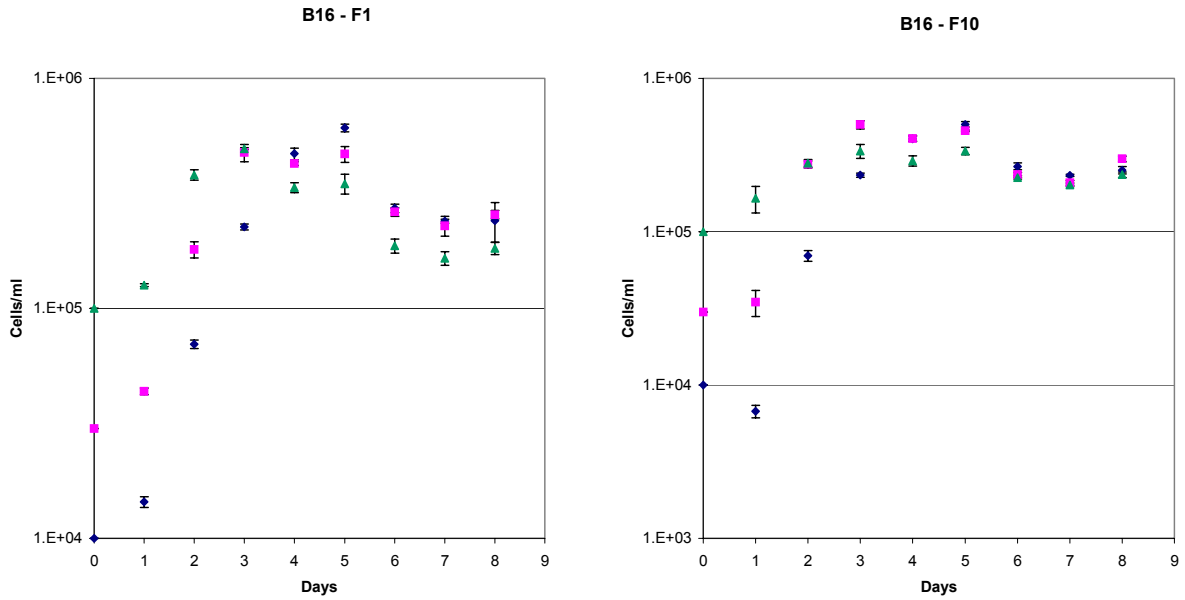


Fig. 3.1 Growth curve of B16-F1 and B16-F10 mouse melanoma cells. ◆1 x 10^4, ■ 3 x 10^4, ▲ 1 x 10^5 cells/ml

Seeding density	B16-F1	B16-F10
1 x 10^4 cells/ml	12 h	9 h
3 x 10^4 cells/ml	14 h	12 h
1 x 10^5 cells/ml	36 h	47 h

Tab. 3.1 Population doubling times of B16 mouse melanoma cells as a function of seeding density

Both cell lines secreted melanin to the cell culture medium (see **picture 6**), a phenomenon that is described for B16-F1 cells but not for B16-F10.

Picture 6 On the left: medium of B16 mouse melanoma culture three days after seeding. On the right: medium without cells.

3.2 Cytotoxicity of PUFA

3.2.1 Cytotoxicity on normal human melanocytes

The cytotoxicity of the test substances was determined using the neutral red assay. Cultures of normal human melanocytes were seeded in microtiterplates and incubated with different concentrations of polyunsaturated fatty acids in cell culture medium. After 72 h the viable cells were stained with neutral red. Cell viability in comparison to untreated control was calculated and plotted against concentration of test compound (see **Fig. 3.2**). The two cell lines show a similar response to the test compounds. All test substances led to a very small, dose-dependent reduction of viability in both cell lines.

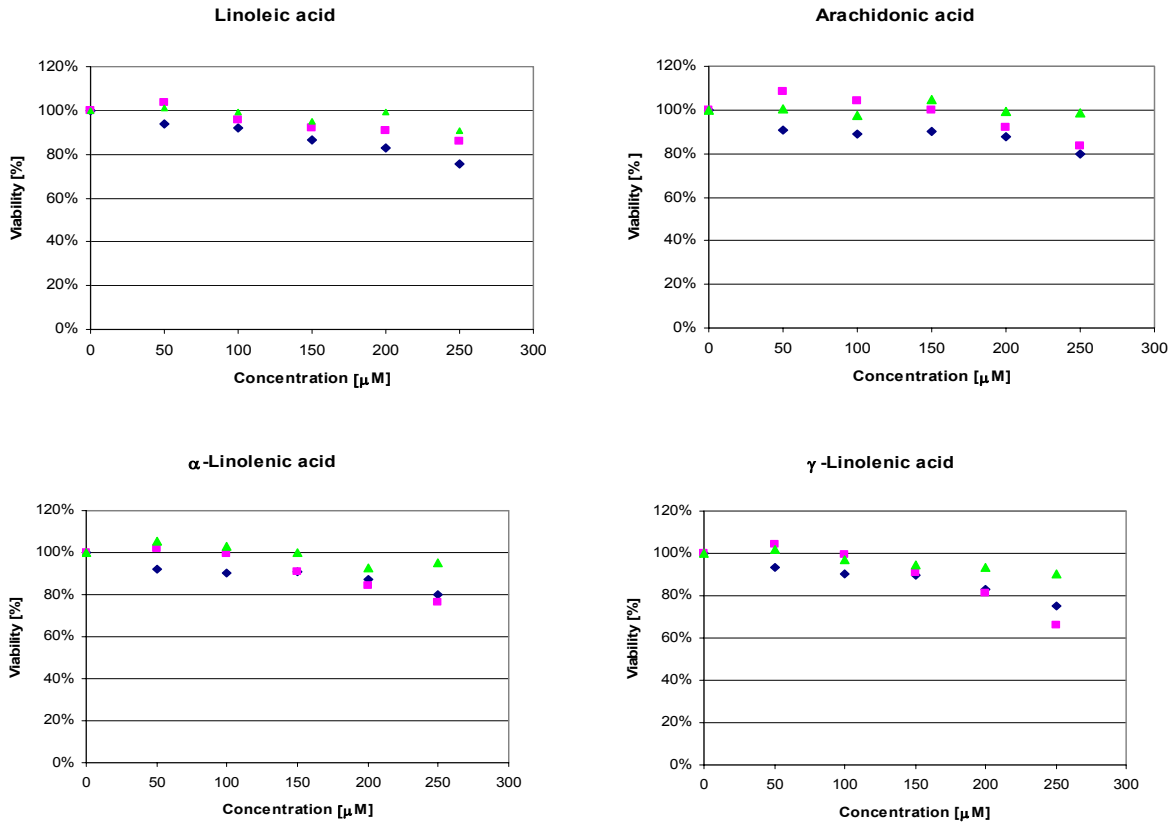


Fig. 3.2 Cytotoxicity of linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid on different cell lines of cultured normal human melanocytes expressed as percent viability of vehicle treated control.

■ NHMP01 passage 15, ■ NHMP01 passage 22, ■ NHM 11 passage 10

3.2.2 Cytotoxicity on mouse melanoma cells

The cytotoxicity of the test substances was determined using the neutral red assay. Cultures of B16-F1 and B16-F10 mouse melanoma cells were seeded in microtiterplates and incubated with different concentrations of polyunsaturated fatty acids in cell culture medium. After 72 h the viable cells were stained with neutral red. Incorporated dye was extracted and quantified with a spectrophotometer at 540 nm. Cell viability in comparison to untreated control was calculated and plotted against concentration of test compund (see **Fig 3.3**). The two cell lines showed a very similar response to the test compounds. All test compounds led to a dose dependent reduction of viability in both cell lines. Incubation with 500 μM of PUFA in medium resulted in a complete loss of viability of B16 mouse melanoma cells.

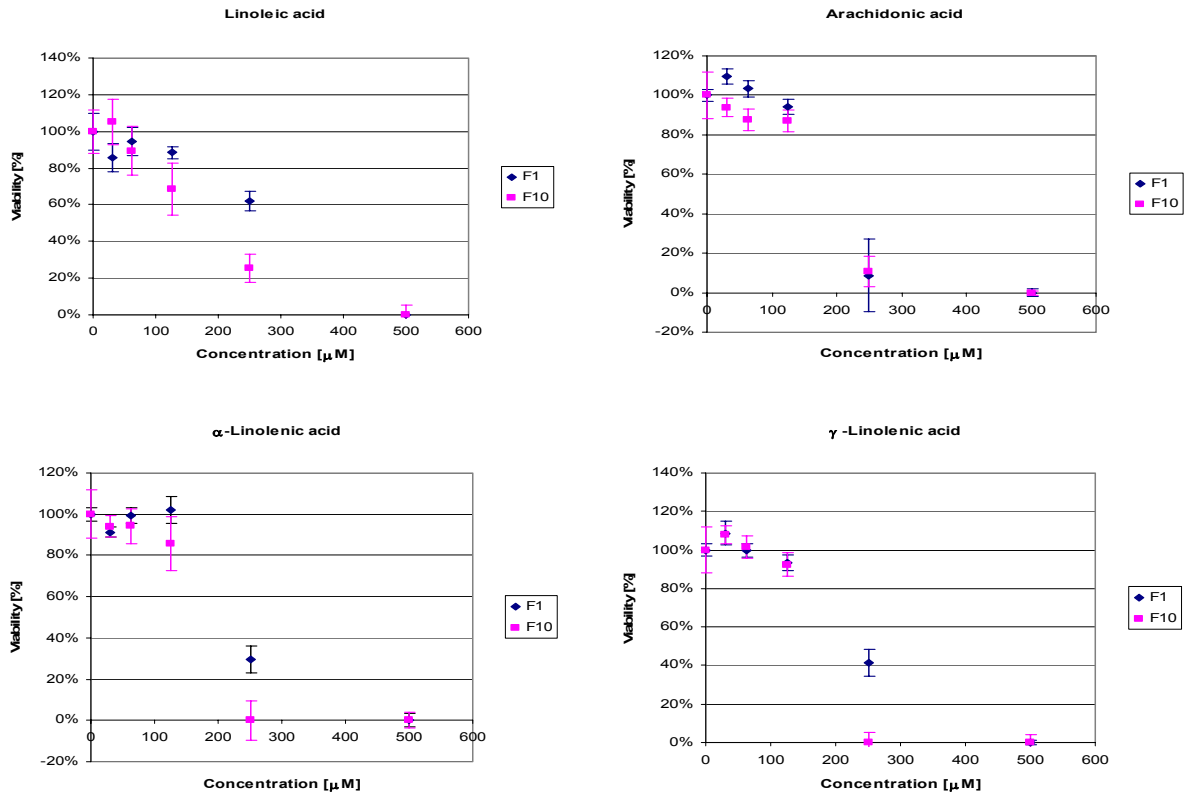


Fig. 3.3 Cytotoxicity of linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid on cultures of B16 mouse melanoma cells. Results are expressed as percent viability compared to vehicle treated control.

The IC_{50}, which is the concentration of compound promoting 50% inhibition of cell viability, was determined by curve fitting using the software TableCurve™. The results are shown in **Table 3.2**.

	B16-F1	B16-F10
Linoleic acid	273 μM	169 μM
Arachidonic acid	184 μM	188 μM
α-Linolenic acid	224 μM	162 μM
γ-Linolenic acid	232 μM	162 μM

Tab. 3.2 IC_{50} values of the four tested polyunsaturated fatty acids for B16-F1 and B16-F10 mouse melanoma cells.

For B16-F10 cells the IC_{50} values of linoleic acid, α-linolenic acid and γ-linolenic acid were lower than for B16-F1. There are only small differences in cytotoxicity beetwen the four polyunsaturated fatty acids while the IC_{50} values of arachidonic acid are identical for the two sublines.

In the experiments with B16-F1 cells linoleic acid, α-linolenic acid and γ-linolenic acid show higher IC_{50} values than arachidonic acid. In contrast for B16-F10 cells the IC_{50} value of arachidonic acid is higher than the values of the remaining PUFAs.

3.3 Effects of PUFA on melanogenesis of mouse melanoma cells

3.3.1 Tyrosinase mRNA content

To elucidate the effect of PUFA on tyrosinase gene expression, the tyrosinase mRNA content of cells treated with 25 μM of test substance for 24 h was determined by real-time TaqMan® PCR. This method was chosen as it is a fast and sensitive one compared with northern blot analysis. As it is known that tumor promoting phorbol esters, such as PMA, can reduce tyrosinase mRNA levels in B16 mouse melanoma cells [71], we used this substance as a positive control for the assay system. Experiments showed that PMA significantly reduced the tyrosinase mRNA level in mouse melanoma cells (see **Fig.3.4**).

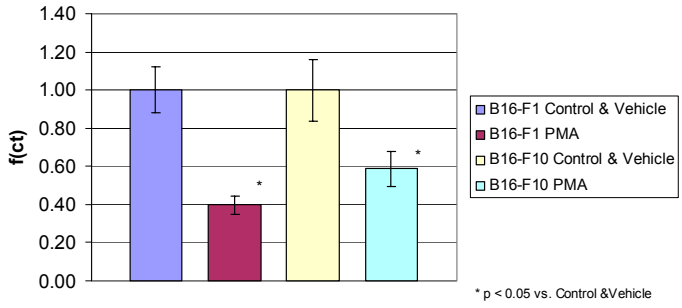


Fig. 3.4 Reduction of intracellular tyrosinase mRNA content of B16 mouse melanoma cells after incubation with 30 nM PMA for 24 hours.
$f(c_t)$ corresponds to the „fold-expression" relative to reference. Data are mean values of triplicate determinations ± SD. ANOVA and Student's t-test were used for statistical analysis.

In B16-F10 cells none of the tested PUFA had an influence on the tyrosinase mRNA level; in B16-F1 cells only linoleic acid and arachidonic acid led to a significant ($p<0.05$) moderate decrease in the tyrosinase mRNA level (see **Fig. 3.5**).

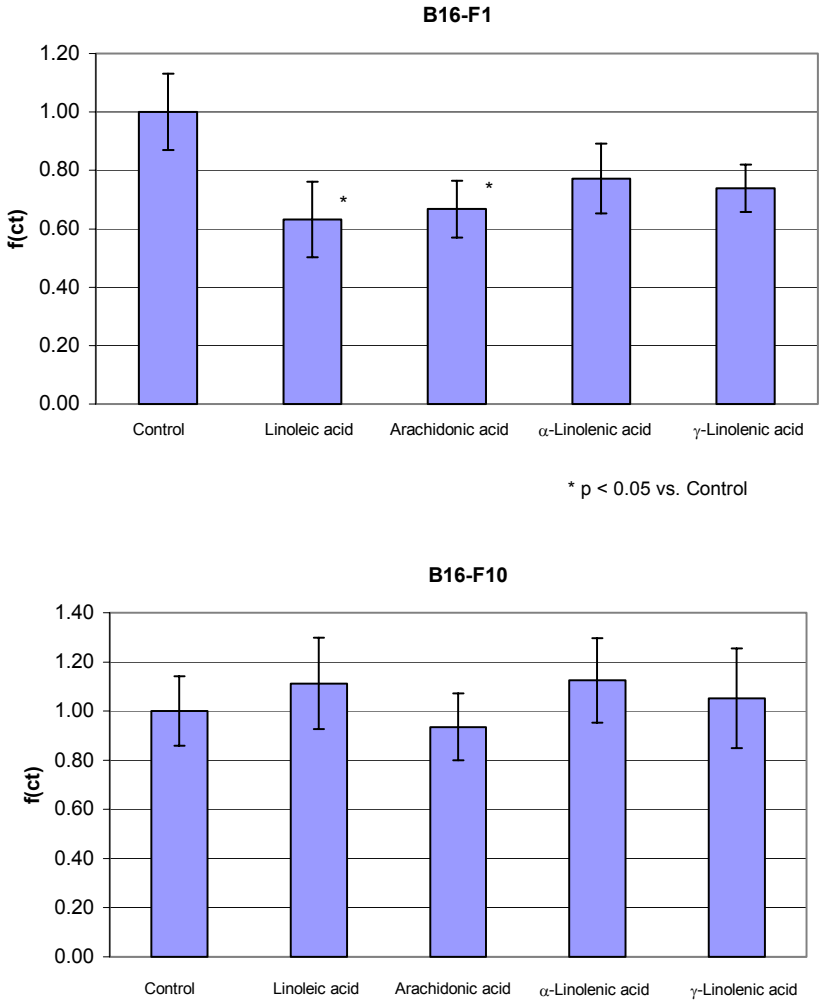


Fig. 3.5 Influence of 25 μM polyunsaturated fatty acids on tyrosinase mRNA content of B16 mouse melanoma cells. Incubation time was 24 h.
$f(c_t)$ corresponds to the „fold-expression" relative to reference. Data are mean values of triplicate determinations ± SD. ANOVA and Dunett test were used for statistical analysis.

3.3.2 Tyrosinase activity

DOPA oxidase activity of tyrosinase in crude cell extracts from PUFA treated B16 mouse melanoma cells was determined using a modified version of the method described by Ando [58]. In first experiments it was shown that, in contrast to the results of Ando, the reaction was not linear. Therefore the cell extract was diluted. With the diluted extract the reaction was linear over a period of 10 min.

In both sublines, B16-F1 and B16-F10, none of the tested PUFA leads to a decreased tyrosinase activity. Even after a 72 h incubation with test substance, the tyrosinase activity was not affected (see **Fig. 3.6**).

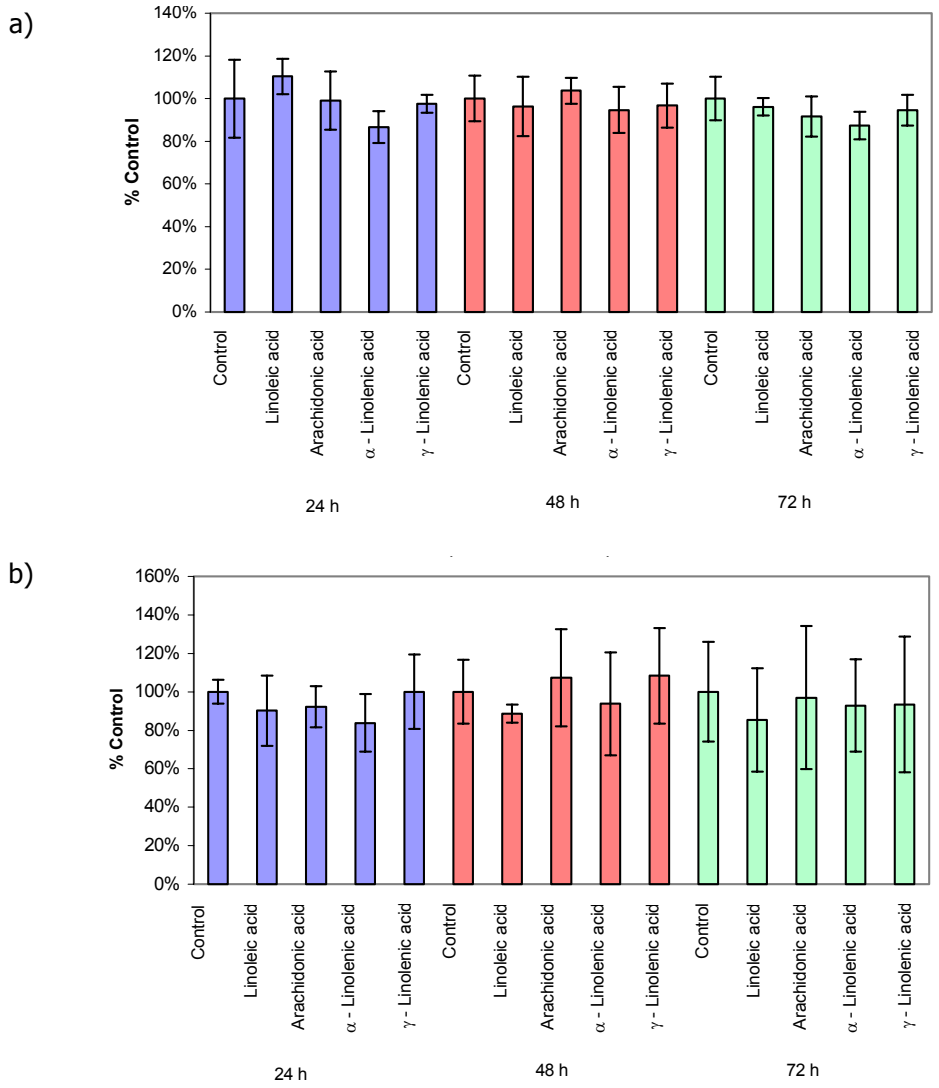
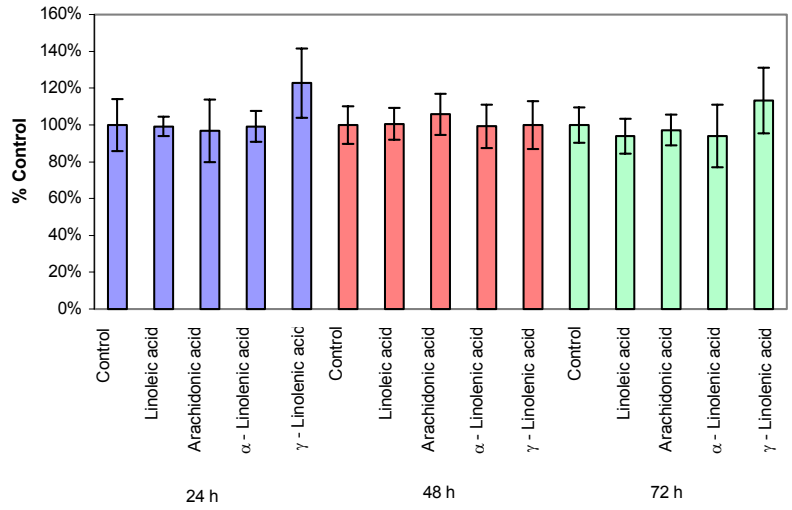


Fig. 3.6 Influence of 25 µM polyunsaturated fatty acids on tyrosinase activity of B16 mouse melanoma cells after treatment period of 24, 48 and 72 hours. Tyrosinase activity was measured according to a modified method of Ando [58].
Data are expressed as percent of vehicle treated control and are mean values of twelve individual values ± SD. a) B16-F1; b) B16-F10

In addition to the method of Ando a second assay to measure the DOPA oxidase activity of tyrosinase was performed. With this assay too, no reduction of tyrosinase activity of mouse melanoma cells upon treatment with PUFA could be detected (see **Fig. 3.7**).

a)



b)

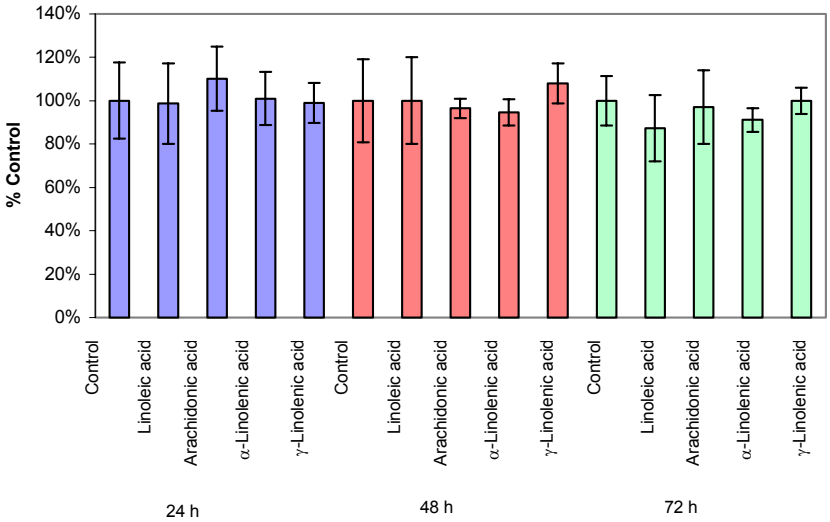


Fig 3.7 Influence of 25 μM polyunsaturated fatty acids on tyrosinase activity of B16 mouse melanoma cells after treatment period of 24, 48 and 72 h.
Tyrosinase activity was measured according to a modified method of Winder [67].
Data are expressed as percent of vehicle treated control and are mean values of twelve individual values ± SD. a) B16-F1; b) B16-F10

3.3.3 Melanin content

The potential of PUFA to reduce the intracellular melanin content of B16-F1 mouse melanoma cells is well known from the work of Ando. As there exist different sublines of B16 mouse melanoma cells, it was of interest to investigate whether there are differences in their response to the treatment with PUFA.

Melanin from cells grown in the presence of PUFA for 24, 48 and 72 h was extracted and quantified spectrophotometrically. For results see **Fig. 3.8**.

In both sublines the intracellular melanin content was reduced in a time-dependent manner by all of the tested fatty acids. α-Linolenic acid and γ-linolenic acid were more potent in their effects on melanin content. It was also shown that in B16-F10 cells, the test substances led to a stronger reduction of the melanin content than in B16-F1 cells.

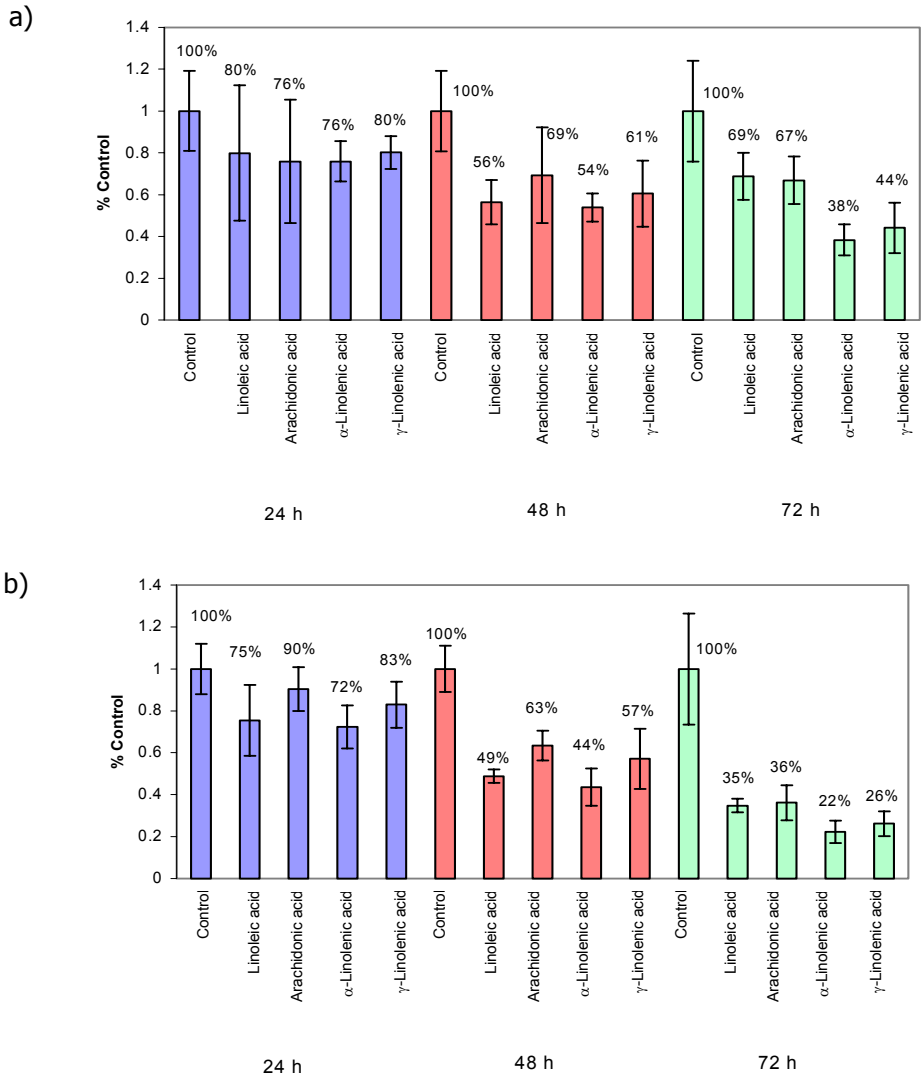


Fig. 3.8 Influence of 25 μM polyunsaturated fatty acids on intracellular melanin content of B16 mouse melanoma cells after a treatment period of 24, 48 and 72 h.
Data are expressed as percent of vehicle treated control and are mean values of twelve individual values ± SD. a) B16-F1; b) B16-F10

In the first experiments with PUFAs and mouse melanoma cells it was observed that medium from treated cells darkened much faster than medium from the untreated control. To examine this effect, secreted melanin in the medium and intracellular pigment were measured in the same experiment.

Surprisingly, it was shown that, in both sublines, the treatment with 25 μM of PUFA led to a significant increase of melanin in medium after 24 h (see **Fig. 3.9**).

With time this discrepancy between untreated and treated cells in secretion of melanin to the medium was reduced. In B1-F1 cells it occurred very fast, while in the B16-F10 subline, secretion of melanin to the medium was enhanced by arachidonic and α-linolenic acid even after a treatment period of 72 h.

All of the tested polyunsaturated fatty acids showed a time-dependent inhibitory effect on intracellular melanin content of B16 mouse melanoma cells.

It is apparent that a high amount of total melanin produced is secreted to the medium. The proportion of melanin secreted to the medium is greater in cultures of B16-F1 cells. In contrast B16-F1 cells have a lower intracellular melanin content as compared with B16-F10 cells.

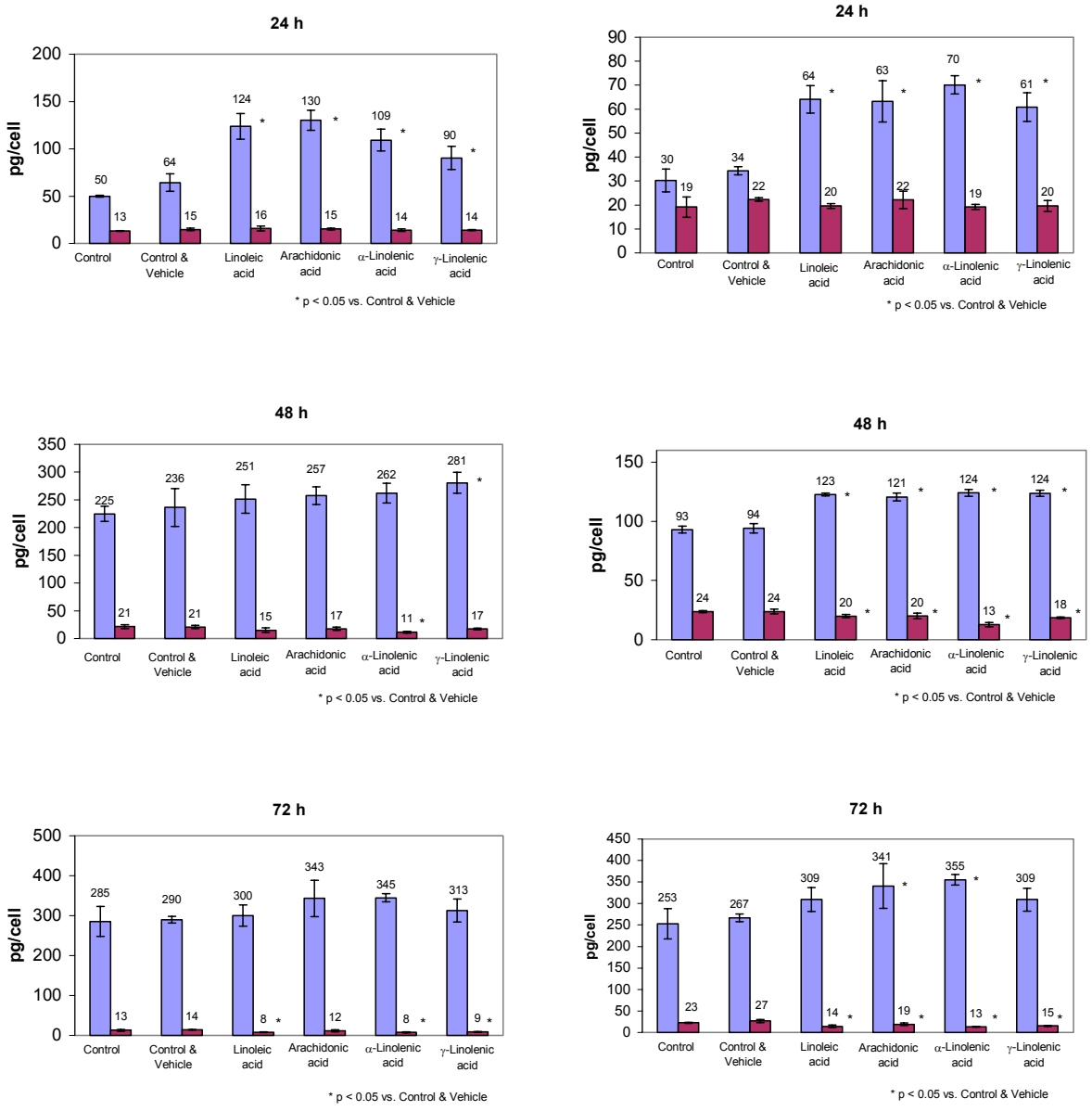


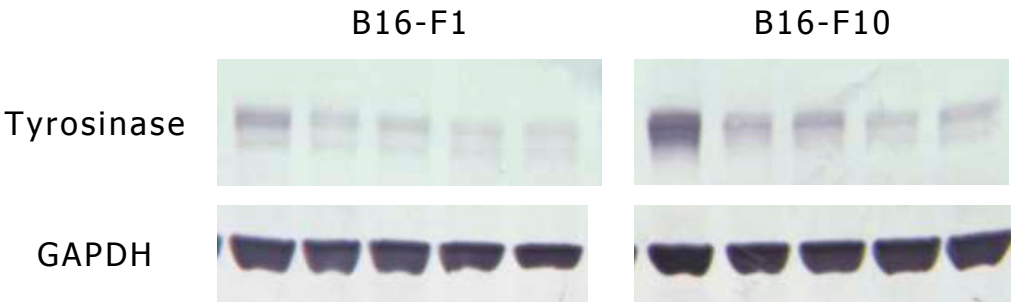
Fig. 3.9 Intra- and extracellular melanin content of B16-F1 (left) and B16-F10 (right) mouse melanoma cells after treatment with 25 μM of PUFA for 24, 48 and 72 h. Data are expressed as picogram melanin per cell and are mean values of quadruplicate determinations ± SD. Dunnet test was used for statistical analysis of the data.

3.3.4 Tyrosinase content

Measurement of the tyrosinase content of cells treated with PUFA was performed by Western Blot and ELISA method.

Western Blot

Cultures of B16-F1 and B16-F10 mouse melanoma cells were incubated with 25 μM of PUFA for 72 h. After this incubation cells were harvested and proteins extracted. The protein extracts were separated by SDS polyacrylamide gel electrophoresis. The gel was blotted on a PVDF membrane and tyrosinase was specifically detected using a polyclonal goat anti mouse/rat tyrosinase antibody and a rabbit anti goat antibody labeled with alkaline phosphatase. In the first experiments bands were very difuse and difficult to interprete. Optimization of the transfer conditions led to better results. At the same time the decision was taken to detect the protein GAPDH on the blot. This so-called „house-keeping gene" is often used as an endogenous loading control. With these modifications of the protocol it was possible to detect a decrease in tyrosinase content in B16 mouse melanoma cells dependent on the treatment with PUFA (see **picture 7**).



Picture 7 Western blot analysis of tyrosinase and GAPDH from cultured B16-F1 and B16-F10 mouse melanoma cells after treatment with 25 μM polyunsaturated fatty acids for 72 h. The lanes from left to right indicate the control, linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid.

The blots were analyzed densitometrically and the results (see **Fig. 3.11**) show that treatment with 25 μM of PUFA led to a reduced tyrosinase content that is characterized by a reduced density of tyrosinase bands of cells treated with polyunsaturated fatty acids.

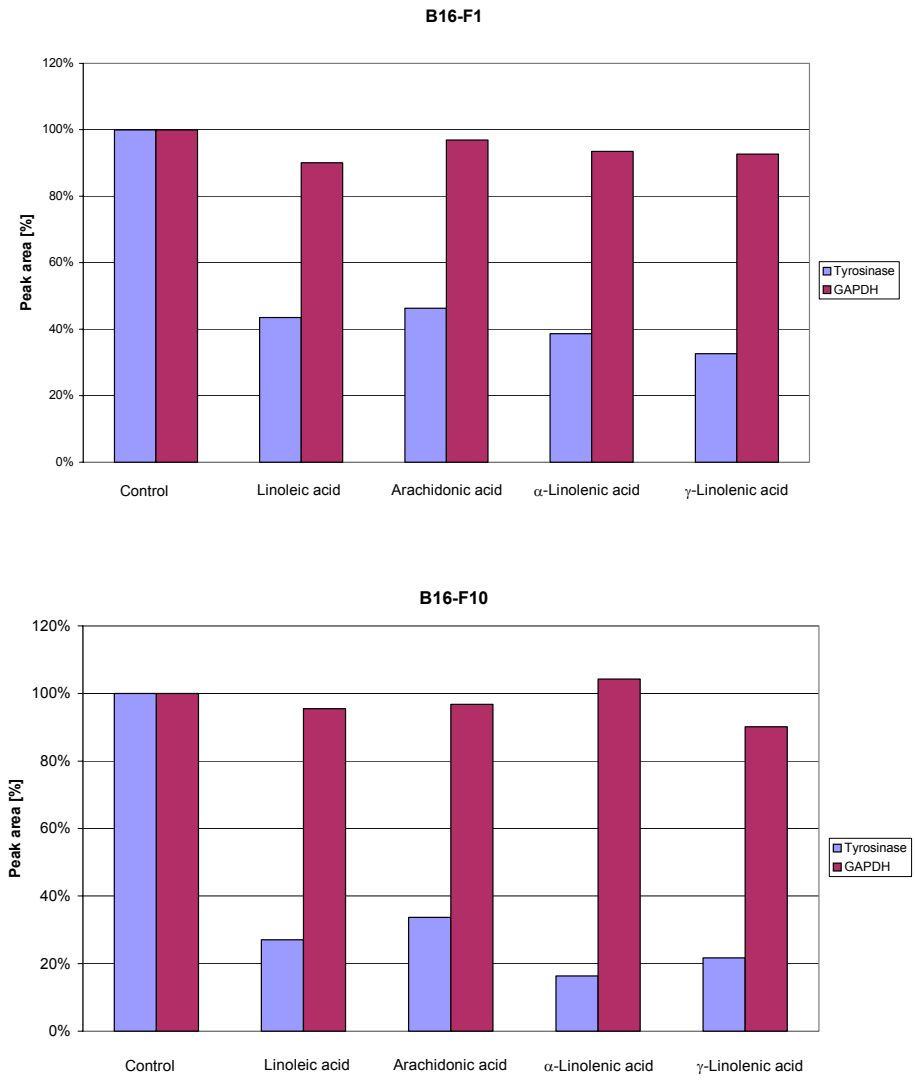


Fig. 3.11 Densitometrical analysis of western blot of tyrosinase and GAPDH of B16-F1 and B16-F10 cells treated with 25 μM of PUFA for 72 h. Data are expressed as percentage of peak area of control.

ELISA

With the ELISA method a significant decrease of tyrosinase content of mouse melanoma cells treated with 25 μM PUFA for 72 h, was observed for all of the tested substances (see **Fig. 3.12**). In B16-F10 cells the reduction was not as dramatic as in B16-F1 subline. Differences beetwen the four tested fatty acids in their potential to reduce tyrosinase content were not obvious.

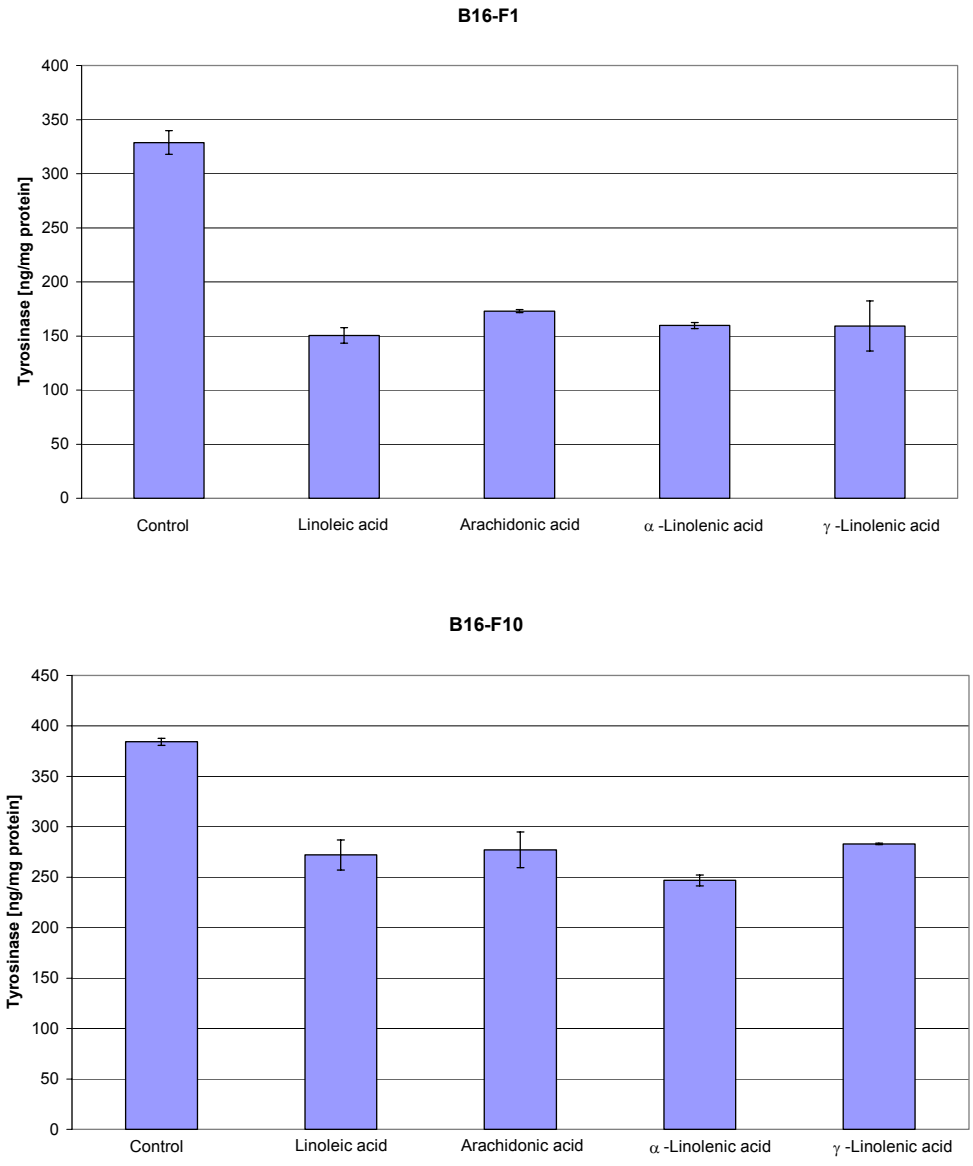


Fig. 3.12 Tyrosinase content of B16-F1 and B16-F10 cells after treatment with 25 μM PUFA for 72 h measured with ELISA. Data are expressed as nanogramm tyrosinase per milligramm protein and are mean values of duplicate determinations ± SD.

3.4 Effects of PUFA on melanogenesis of normal human melanocytes

3.4.1 Tyrosinase mRNA content

As for B16 mouse melanoma cells, a specific assay to measure the tyrosinase mRNA content was also developed for human melanocytes. Cells incubated with 20 μM β-estradiol in themedium for 24 hours showed a significant increase in the tyrosinase mRNA content as measured by real-time TaqMan™ PCR. This effect of β-estradiol on tyrosinase mRNA content of cultured human melanocytes is well known and was examined and published by Kippenberger et al. [41].
In contrast, the tyrosinase content of cells cultivated in presence of 100 μM PUFA for 24 h was not significantly altered as compared with the untreated control (see **Fig. 3.13**).

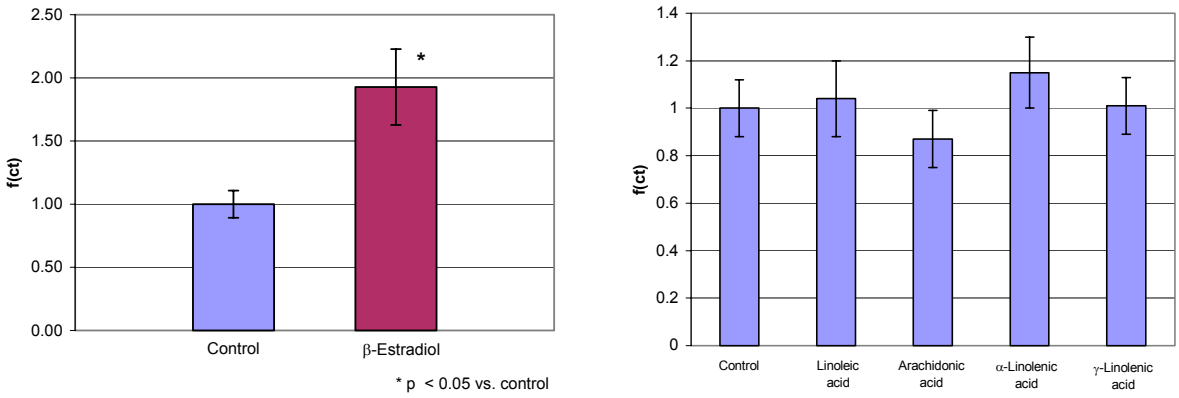


Fig. 3.13 Influence of 20μM β-Estradiol and 100 μM PUFA on tyrosinase mRNA content of cultured human melanocytes. The treatment period was 24 h and data are mean values of triplicate determinations ± SD. ANOVA and Student's t-test were used for statisical analysis of the data.
$f(c_t)$ corresponds to the „fold-expression" relative to reference.

3.4.2 Tyrosinase activity

The four polyunsaturated fatty acids linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid were tested for their ability to reduce tyrosinase activity in cultured human melanocytes. Test substances in ethanol as well as controls were therefore applied to the medium. The cells were cultured in the presence of these substances for 72 h. DOPA oxidase activity of tyrosinase was not affected by any of the polyunsaturated fatty acids (see **Fig. 3.14**). The experiment was also performed with other cell lines of NHM showing very similar results.

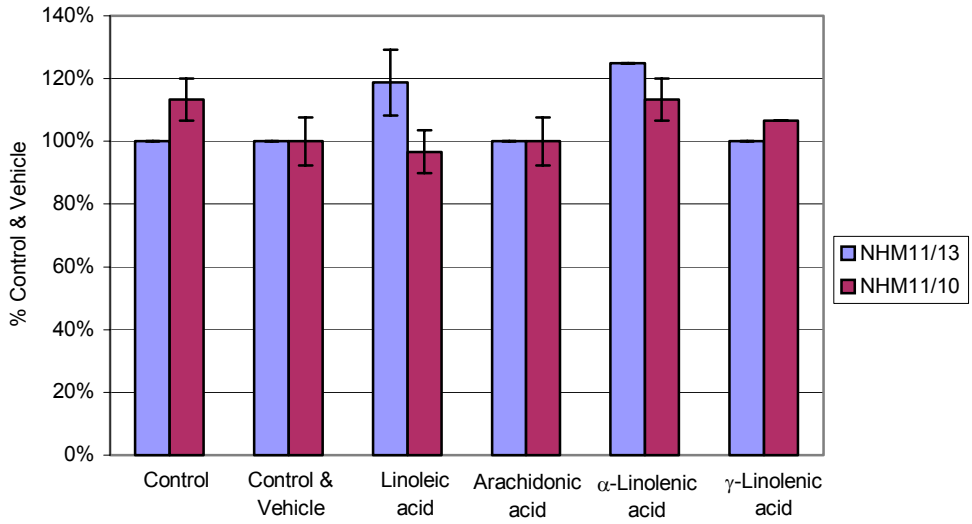
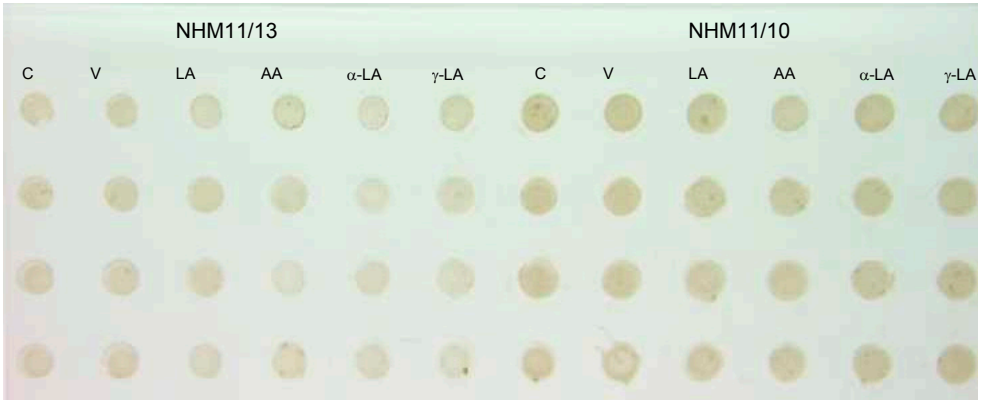


Fig. 3.14 Tyrosinase activity of cultured human melanocytes after treatment with 100 μM PUFA for 72 h. Tyrosinase activity was determined according to the method of Winder [67]. Data are expressed as percentage of vehicle treated control and are mean values of quadruplicate determinations ± SD.

3.4.3 Melanin content

PUFAs were applied to cultures of NHM to examine their influence on the melanin content. Cells grown for 72 h in the presence of fatty acids were harvested and blotted on a nitrocellulose membrane (see **picture 8**). The air-dried membrane was photographed with a digital camera and the spots were analyzed densitometrically (see **Fig. 3.15**). In correspondance to the results of the mRNA and tyrosinase activity none of the substances had an influence on the melanin content. These results were confirmed by experiments with other cell lines of NHM.



Picture 8 Human melanocytes treated with 100 μM of PUFA for 72 h blotted on a nitrocellulose membrane. On the left: cells from the cell line NHM11 at passage 13; on the right: the same cell line at passage 10.
C = control, V = vehicle treated control, LA = Linoleic acid, AA = Arachidonic acid, α-LA = α-Linolenic acid, γ-LA = γ-Linolenic acid

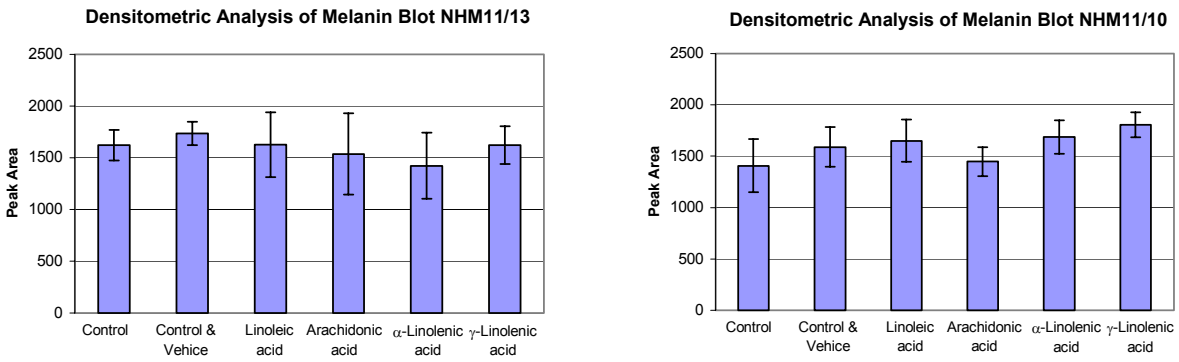


Fig. 3.15 Densitometric analysis of blotted human melanocytes that were treated with 100μM PUFA for 72 h. Data are expressed as peak area of the dots and are mean values of quadruplicate determinations ± SD.

3.4.4 Tyrosinase content

Western Blot

Crude cell extracts from human melanocytes treated with 100 μM of PUFA for 72 h were separated by SDS polyacrylamide gel electrophoresis and proteins were blotted on PVDF membrane. Tyrosinase and GAPDH (as an internal loading control) were detected with specific antibodies. Tyrosinase bands showed a molecular weight of about 75 kDa and GAPDH of about 36 kDa.

PUFAs had no effect on the tyrosinase content of human melanocytes. There are differences in intensity of bands of tyrosinase (see **picture 9**) but densitometrical analysis (**Fig. 3.16**) showed that intensity of tyrosinase and GAPDH bands correlate very well, so the differences in intensity may have occured by pipetting variations.

Picture 9 Western blot analysis of tyrosinase and GAPDH from cultured human melanocytes after treatment with 100 μM polyunsaturated fatty acids for 72 h. The lanes from left to right indicate the control, linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid.

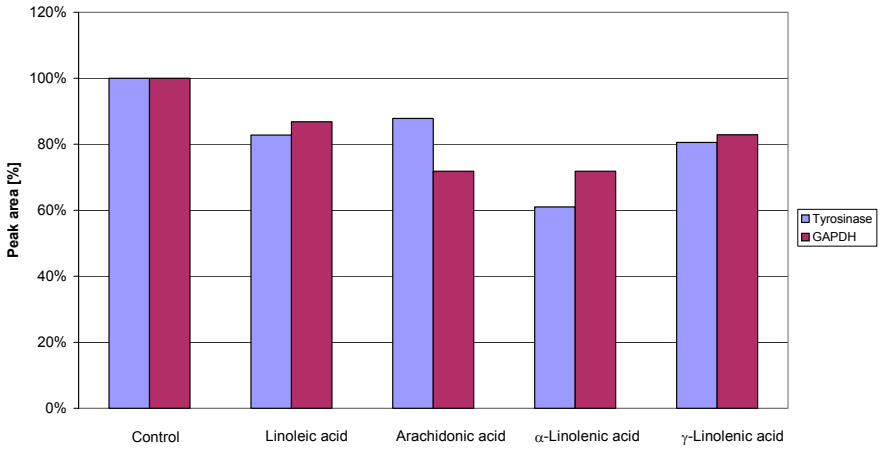


Fig. 3.16 Densitometrical analysis of western blot of tyrosinase and GAPDH of human melanocytes treated with 100 μM of PUFA for 72 h. Data are expressed as percentage of peak area of control.

ELISA

Alternatively the tyrosinase content of crude cell extracts of human melanocytes treated with PUFA for 72 h was determined using ELISA method.

The tyrosinase content was dramatically reduced by all of the four polyunsaturated fatty acids. Linoleic acid led to a reduction of about 65% while arachidonic, α-linolenic and γ-linolenic acid led to a reduction of about 30% of tyrosinase content (see **Fig 3.17**).

The results of this experiment were confirmed by an additional experiment that showed similar results.

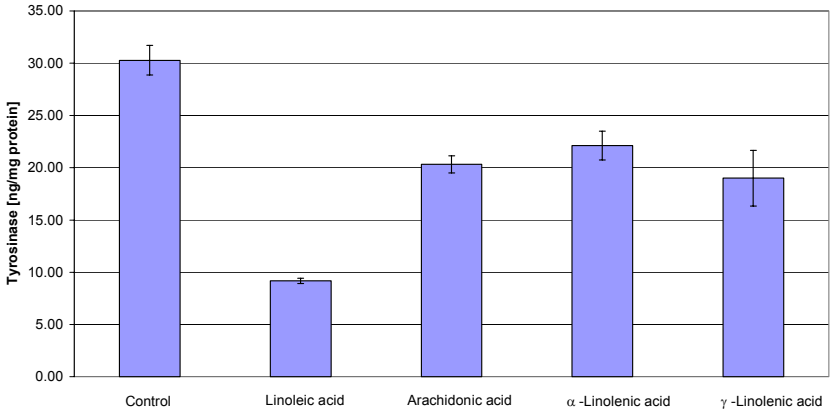
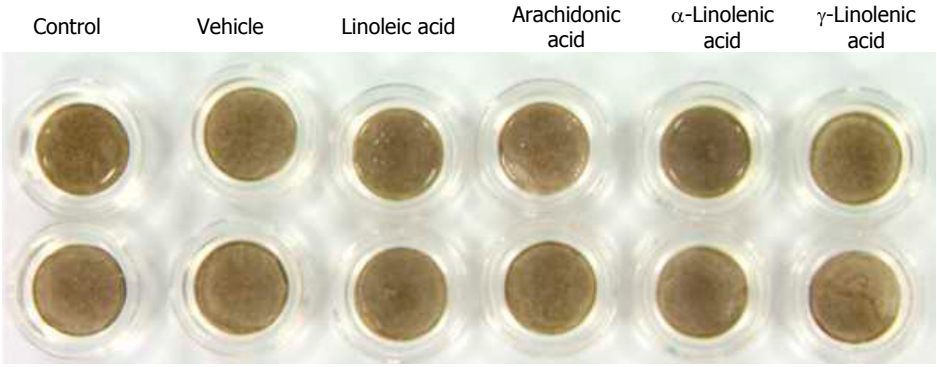


Fig. 3.17 Tyrosinase content of human melanocytes after treatment with 100 μM PUFA for 72 h measured with ELISA. Data are expressed as nanogramm tyrosinase per milligramm protein and are mean values of duplicate determinations ± SD.

3.5 Effects of PUFA on pigmentation of MelanoDerm™

To elucidate the question if PUFAs have an influence on the pigmentation of human melanocytes cultured in a more *in vivo* like manner, specimens of a pigmented reconstituted 3D epidermal model were treated with 1% (v/v) of PUFA in 10% ethanol (v/v) in WFI. After a treatment period of three weeks no lightening of the tissues was observed (see **picture 10**).



Picture 10 MelanoDerm™ tissues after a three week treatment with 1% of PUFA in 10% of ethanol or 10% of ethanol alone (vehicle) or no treatment at all (control).

The densitometrical analysis of the macroscopic picture of the tissues showed no difference in pigmentation between treated and untreated specimens (**Fig. 3.18**).

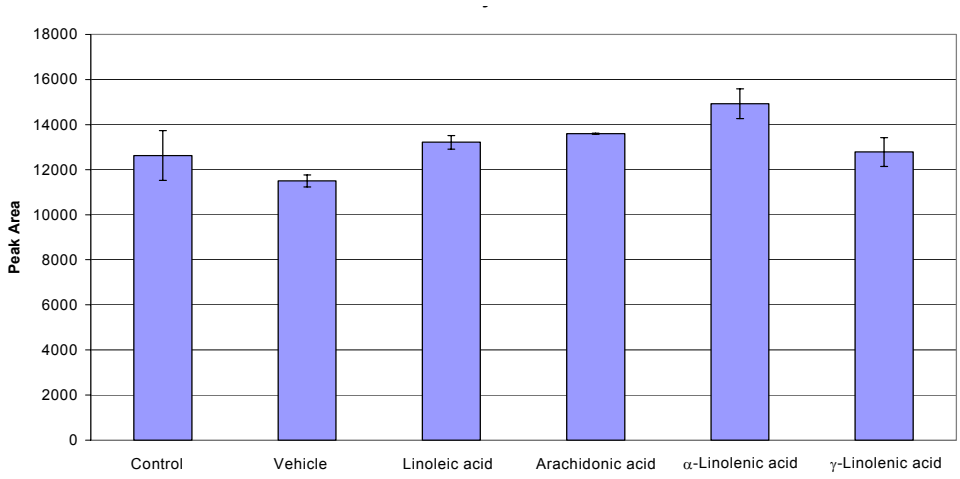


Fig. 3.18 Densitometrical analysis of MelanoDerm™ tissues treated for three weeks with 1% of PUFA in 10% of ethanol or 10% of ethanol alone (vehicle) or no treatment at all (control). Data are expressed as peak area and are mean values of duplicate determinations ± SD.

The optical density of melanin extracted from the tissues was measured at 405 nm (**Fig. 3.20**). There was no alteration of the melanin content by treatment with PUFA.

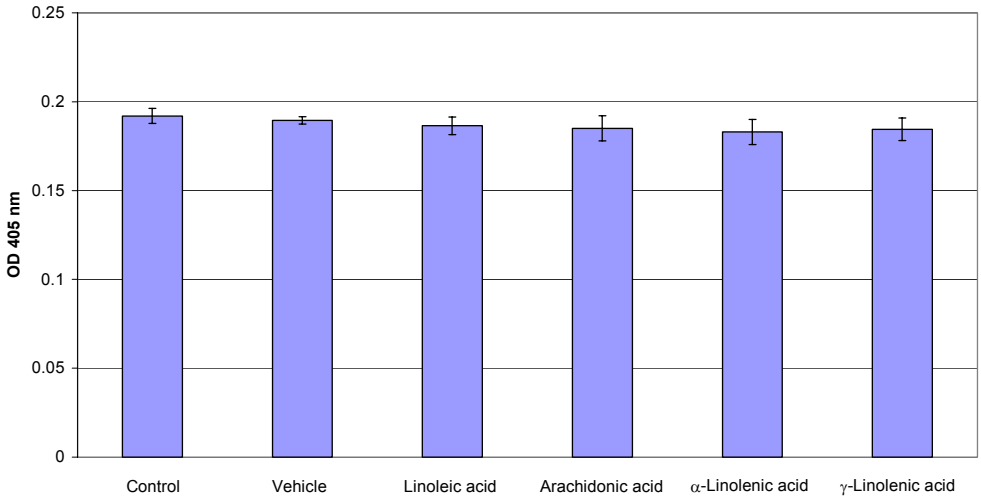
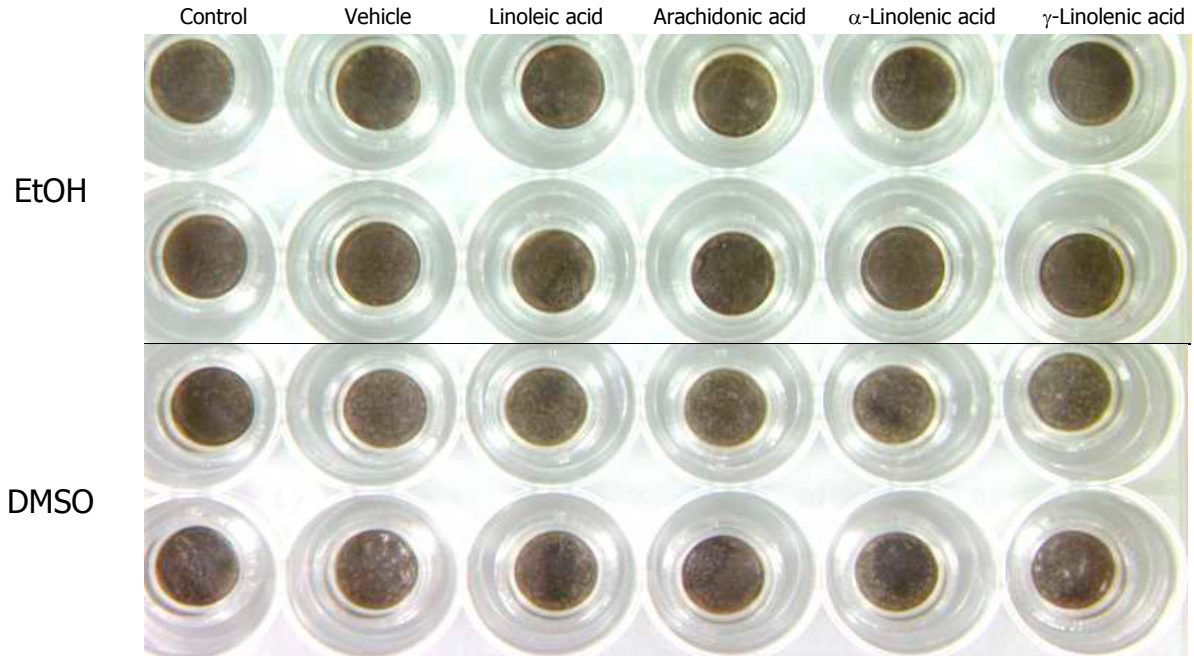


Fig 3.20 Optical density of extracted melanin from MelanoDerm™ tissues treated for three weeks with 1% of PUFA in 10% of ethanol or 10% of ethanol alone (vehicle) or no rteatment at all (control). Data are mean values of duplicate determinations ± SD.

In a second experiment the influence of a higher concentration of PUFA on pigmentation of MelanoDerm™ tissues was examined. Specimens of the 3D epidermis model were treated with 2.5% (v/v) of unsaturated fatty acid in 25% (v/v) of ethanol or 25% (v/v) of DMSO in WFI for three weeks.

After this treatment period no lightening of the tissues could be observed (**picture 11**). Densitometrical analysis of the macroscopic picture of the tissues neither showed any difference in pigmentation of treated and untreated specimens (**Fig. 3.21**). Additionally spectrophotometrical analysis of extracted melanin did not show any alteration in melanin content after treatment with polyunsaturated fatty acids (**Fig. 3.22**).



Picture 11 MelanoDerm™ tissues after a three week treatment with 2.5% of PUFA in vehicle or vehicle or no treatment at all (control). Vehicle consisted of 25% (v/v) of ethanol or 25% (v/v) of DMSO in WFI.

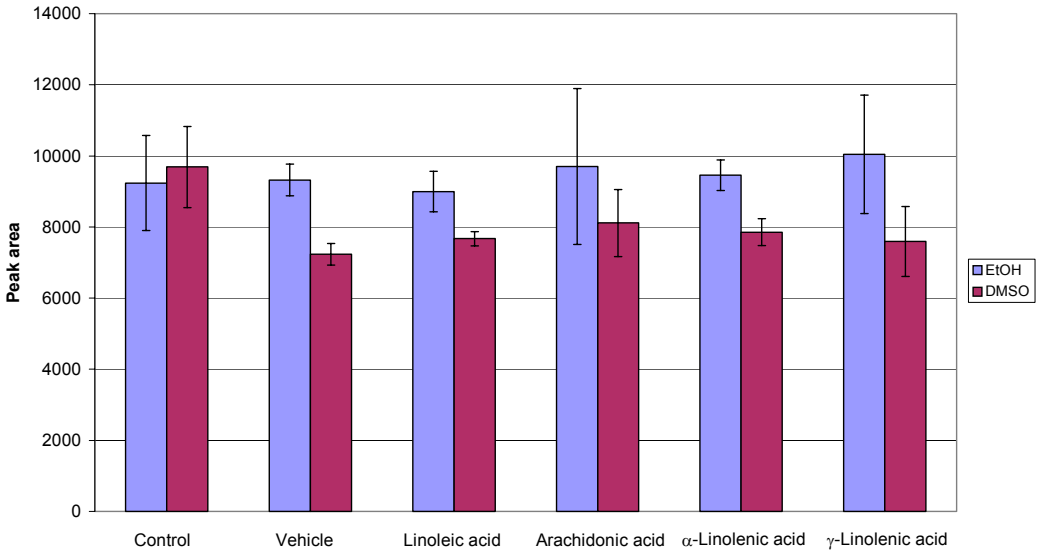


Fig 3.21 Densitometrical analysis of MelanoDerm™ tissues treated for three weeks with 2.5% of PUFA or vehicle or no treatment at all (control). Vehicle consisted of 25% (v/v) EtOH or 25% (v/v) DMSO in WFI. Data are expressed as peak area and are mean values of duplicate determinations ± SD.

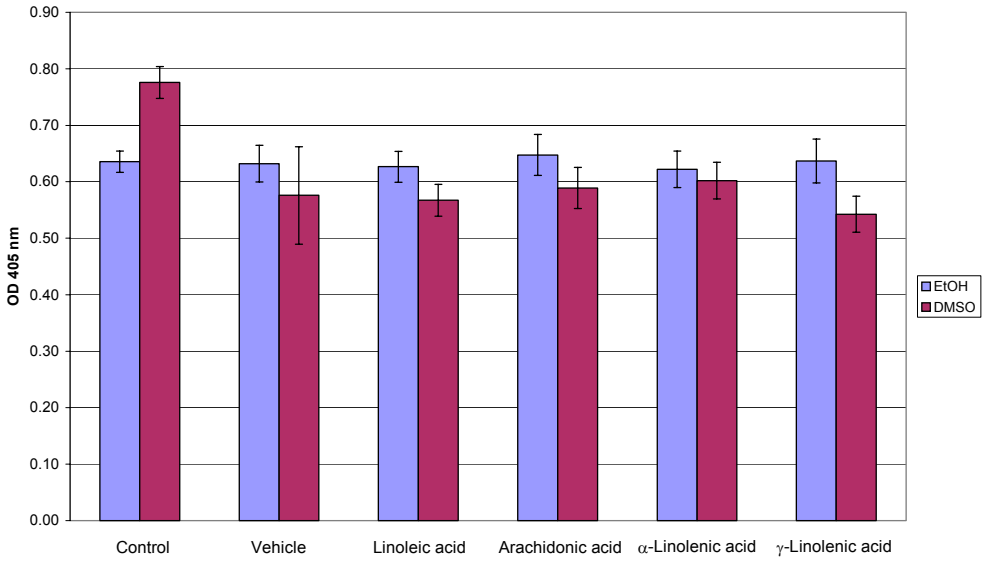


Fig 3.22 Optical density of extracted melanin from MelanoDerm™ tissues treated for three weeks with 2.5% of PUFA in vehicle or vehicle alone or no treatment at all (control). Vehicle consisted of 25% (v/v) of EtOH or 25% (v/v) of DMSO in WFI. Data are mean values of duplicate determinations ± SD.

4. Discussion

4.1 B16 mouse melanoma cells

B16 mouse melanoma cells were chosen as an *in vitro* model to investigate the influence of the polyunsaturated fatty acids linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid on pigmentation.

Mouse melanoma cells of the B16 cell line are widely used in studies of the regulatory mechanisms of melanogenesis [72] because these cells have a short population doubling time and produce, unlike the vast majority of commercially available human melanoma lines, ample melanin during serial passage of the cells.

Various sublines of this cell line exist: the European collection of cell cultures lists three, the American type culture collection four and the Riken cell bank five different sublines. It seems that there are further sublines that are not commercially available.

Reports about variations between these sublines in melanogenic activity and responsiveness to agents modulating melanogenesis do not exist. One can speculate whether such differences do not exist, or whether no studies were done about this topic. In this work it could be shown for the first time that such variations in the endogenous melanogenic activity and in the reaction to melanogenesis modulating compounds between B16-F1 and B16-F10 subline do exist.

It appears that the export of melanin from the cell is enhanced in the B16-F1 subline compared to the B16-F10 subline. Under normal culture conditions B16-F1 cells secrete 80 to 95% of the melanin produced to the medium, while for B16-F10 cells the amount of secreted melanin ranges from 60 to 90%. With increasing cultivation time the secreted melanin accumulates in the medium and therefore the amount of extracellular melanin increases too.

Cultures of B16-F1 subline show twentyfour hours after seeding an increase of extracellular melanin from 50 pg/cell compared to 285 pg/cell after a culture time of seventytwo hours, while the intracellular melanin content is quite stable and varies only from 13 to 21 pg/cell.

In contrast the intracellular melanin content of B16-F10 cells is slightly higher and varies from 19 to 24 pg/cell, while the content of extracellular melanin increases from 30 pg/cell to 253 pg/cell over a culture period of seventytwo hours.

Very little is known about the factors that control the secretion of melanin in B16 mouse melanoma cells. It was shown that in cultures of B16/C3 cells serum is required to induce the secretion of melanin to the medium [73]. If these cells were grown in a serum-free, hormone-supplemented medium, melanin was produced after induction of melanogenesis with MSH or dibutyryl cAMP, but was not secreted to the medium. It was speculated that a serum factor that is non dialyzable and can be partially purified by Sephadex chromatography is responsible for the regulation of melanin secretion. Surprisingly this factor was not further characterized. Other factors that may play a role in the regulation of melanin secretion to the extracellular space are calcium [74] and citrate [66].

The results from our work show that the polyunsaturated fatty acids linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid are capable of increasing the secretion of melanin to the medium. Additionally to this stimulation of melanin secretion the intracellular melanin content is decreased by the polyunsaturated fatty acids.

From the data obtained it is obvious that dependent on the subline the tested polyunsaturated fatty acids exhibit differences in the potency of reduction of intracellular melanin content. In cells of the B16-F10 subline the influence of fatty acids is more potent on intracellularly stored melanin compared to the B16-F1 subline.

In contrast to the results of Ando [58] the inhibition of melanogenesis by PUFAs is not correlated with the number of unsaturated bonds, as arachidonic acid that has four unsaturated bonds inhibited melanogenesis in an equally effective manner as linoleic acid that contains only two double bonds.

In both sublines the unsaturated fatty acids that contain three double bonds, α-linolenic acid and γ-linolenic acid, inhibited melanogenesis most effectively. One may be surprised that although arachidonic acid contains the largest number of unsaturated bonds among the fatty acids tested, it does not show the most inhibitory effect on melanogenesis. This was expected from the results of a study where the pharmocological effect of linoleic acid was reversed by adding the antioxidant vitamin E and which suggested that unsaturated bonds correlate with the inhibitory effect on melanogenesis [75].

However, saturated fatty acids, such as palmitic acid and stearic acid, accelerate melanogenesis [57], suggesting that unsaturated bonds are not the only factor that affect melanogenesis of murine cells *in vitro*. In organ-cultured guinea pig skin, arachidonic acid shows a totally different action as it stimulates melanogenesis [76]. However, this induction of melanogenesis is caused by an increase in tyrosinase-positive melanocytes and not by an increase in melanin production of the single melanocyte. This increase of tyrosinase-positive melanocytes may be caused by a stimulation of melanocyte proliferation as it is known that the arachidonic acid metabolites leukotriene C4 and D4 are capable of stimulating melanocyte proliferation *in vitro* [77].

One can speculate that between the stimulation of melanin transport to the extracellular space and the reduction of intracellularly stored pigment, a causal connection exists and that the enhanced secretion of melanin is caused by a change in membrane fluidity mediated by the polyunsaturated fatty acids.

Today it is known that diets deficient in linoleic acid, or that comprise unusual ratios of linoleic acid to α-linolenic acid induce changes in the PUFA composition of neuronal and glial membranes and lead to modifications in membrane fluidity, in the activities of membrane-associated, functional proteins (transporters, receptors, enzymes), and in the production of important signaling molecules from oxygenated linoleic and α-linolenic acid derivatives [78, 79]. Whether such modifications may also occur in mouse melanoma cells and if they can modulate the secretion of melanin to the medium is not known.

Our results suggest that the decrease in intracellular melanin content of B16 mouse melanoma cells upon incubation with PUFA is not mediated by the acceleration of melanin secretion because i) there is a delay between the increase in secretion and the decrease in intracellular melanin content and ii) the surplus of melanin secreted upon treatment with PUFA is much larger than the reduction of the intracellular melanin content. Therefore it seems that the acceleration of melanin secretion and the reduction of intracellular melanin content are two distinct effects.

As stated before, an explanation for the increase in extracellular melanin content can be alterations in membrane integrity leading to an exaggerated secretion of melanin. In this case the overall production of melanin must be enhanced too, as the intracellular melanin content is not decreased at the same time.

One may also speculate that the darkening of the medium upon treatment with PUFA is not caused by the secretion of melanin produced intracellularly but by the extracellular production of dopachrome from tyrosine abundant in the medium. This extracellular production of pigment must be catalyzed by tyrosinase.

From our work we know that addition of mushroom tyrosinase to culture medium leads to a rapid darkening of the solution (unpublished results). It is not known if tyrosinase originated from B16 mouse melanoma cells and released to the medium causes the same effect.

The influence of polyunsaturated fatty acids on the intracellular melanin content of B16 mouse melanoma cells is a much more complex story and the mechanisms behind this inhibition of melanogenesis are speculative as some results from our work are in contrast to the results of Ando and co-workers.

Contrary to Ando and co-workers [59] we were not able to detect an inhibitory effect of polyunsaturated fatty acids on tyrosinase activity. Surprised by these findings we verified our results by repeting the experiments several times and using two different assays for measuring the dopa oxidase activity of tyrosinase with the result that no reduction of the activity of the enzyme could be detected.

In accordance to Ando [61] results from our western blotting and ELISA experiments show that tyrosinase protein levels of B16 mouse melanoma cells were decreased by the polyunsaturated fatty acids after a seventytwo hour incubation. These findings do not correlate with our results from the tyrosinase activity assays. Possible explanations for this potential contradiction in our results are:

1. The antibody used for detection of tyrosinase does not bind to all isoforms of tyrosinase [80, 81] and therefore detects only a proportion of the overall tyrosinase present in the cells.
2. The tyrosinase content of cells treated with polyunsaturated fatty acids is lowered but the catalytic activity of the enzyme is increased.
3. The two explanations above may explain why there is a reduction in tyrosinase content without affection of tyrosinase activity. It is not yet clear why intracellular melanin should be reduced when there is no reduction in tyrosinase activity. It can be speculated that this reduction may be caused by a disorder of the complex of tyrosinase, TRP1, TRP2 and silverprotein [82] located in the melanosome membrane by an alteration in the membrane fluidity of the organelle.

4. Another explanation may be that the assays for measuring the activity of tyrosinase are not specific for tyrosinase and therefore the results for tyrosinase activity are false. In our opinion it seems to be unlikely that two different assays deliver false results at the same time. The assays are well established and are often used in studies on melanogenesis.

All of the hypotheses listed above are speculative and further work will perhaps enlighten the contradiction of our results.

It has been reported that linoleic acid at concentrations consistent with those for the inhibition of melanogenesis in B16-F10 cells does not alter the cell tyrosinase mRNA levels [60]. For B16-F10 cells the results from our TaqMan™ RT-PCR experiments are in accordance with these findings. For B16-F1 cells our results clearly show a significant reduction of tyrosinase mRNA levels by treatment with linoleic acid and arachidonic acid. The tyrosinase mRNA levels of the cells are also reduced after treatment with α-linolenic acid and γ-linolenic acid but the reduction is not significant when the results are statistically analyzed with the Dunnet test. If Student's t-test is used for statistical analysis of the results, the reduction of tyrosinase mRNA level is significant for linoleic acid, arachidonic acid and γ-linolenic acid but the results for α-linolenic acid are not significant.

Nevertheless it seems that the reduction in tyrosinase protein levels in B16-F1 cells is regulated at the transcriptional level, while in B16-F10 cells the reduction of tyrosinase content is caused by modifications at the translational or post-translational level.

Ando and co-workers suggest, that the reduction of tyrosinase protein levels in B16-F10 cells after treatment with the polyunsaturated fatty acid linoleic acid is caused by an acceleration of the proteolytic degradation of the enzyme. The exact mechanism of this proteolytic degradation remains unclear. It is speculated that in normal human melanocytes some tyrosinase is normally degraded by proteasomes and that in amelanotic melanoma cell lines, tyrosinase failed to reach the melanosome because it was retained in the endoplasmatic reticu-

lum and then degraded [83]. It is not known if under normal culture conditions tyrosinase in B16 murine melanoma cells is degraded by proteasomes.

4.2 Normal human melanocytes

Interestingly no work was done to investigate the effect of polyunsaturated fatty acids on melanogenesis of human melanocytes *in vitro* or *in vivo*, although there exists a patent on conjugated linoleic acid and/or derivatives thereof suitable as a cosmetic ingredient for skin lightening. The activity of this ingredient was proven by a pigmentation assay using B16-F1 murine melanoma cells. Incubation with conjugated linoleic acid for 72 h led to a dramatic reduction in secreted melanin. It is not known if the intracellular melanin content was altered too.

We have chosen two *in vitro* systems to elucidate the effect of the polyunsaturated fatty acids linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid on pigmentation of human melanocytes.

Normal human melanocytes isolated from foreskins and cultured in medium containing serum and the two potent synergystic mitogens for melanocyte growth IBMX and PMA [84] served as a first *in vitro* system. In this system we investigated the influence of 100 μM of the polyunsaturated fatty acids mentioned above on tyrosinase mRNA level, tyrosinase protein content, tyrosinase activity and amount of intracellular melanin of cultured human melanocytes. With one exception, our results show that none of the substances affects the melanogenesis of human melanocytes.

Measuring the tyrosinase protein level of PUFA treated cells with our ELISA assay we found a decrease in enzyme level compared to the untreated control. If the tyrosinase content was investigated with the Western blot method no variation in tyrosinase content between treated and untreated cells could be detected.

We are convinced that the polyunsaturated fatty acids do not affect the tyrosinase protein level of human melanocytes *in vitro*. The results of the ELISA assay have to be interpreted with care as the cells used for the assay suffered from toxic effects of the test substances. It was obvious that with increasing passage number human melanocytes became more susceptible to unsaturated fatty acids. These cytotoxic effects were a big problem in our ELISA experiments with polyunsaturated fatty acids and human melanocytes. It was tried to redo the ELISA experiments and to reproduce our findings with other cell lines of human melanocytes with the result that among the cells incubated with 100 μM of polyunsaturated fatty acids, almost all died within twentyfour hours. After some further attempts the experiments had to be stopped, because it was impossible to obtain a sufficiently high amount of cell material. Due to the low sensitivity of the ELISA, about 3×10^7 cells had to be used for a single experiment.

It is not known yet, why there are such differences in the cytotoxic potential of the four polyunsaturated fatty acids on different cell lines of human melanocytes.

As a monolayer culture of human melanocytes where the cells are forced to proliferate by the addition of the tumor promoting agent PMA and IBMX that increases the intracellular cAMP level, to the medium does not resemble the *in vivo* situation, we have chosen a commercially available reconstituted pigmented epidermis to further investigate the influence of polyunsaturated fatty acids on human melanogenesis.

We decided to use Mattek's MelanoDerm™ system that consists of normal, human-derived epidermal keratinocytes (NHEK) and melanocytes (NHM) which have been cultured to form a multilayered, highly differentiated model of human epidermis that exhibits *in vivo*-like morphological and ultrastructural characteristics which are uniform and highly reproducible. The NHM within co-cultures undergo spontaneous melanogenesis leading to tissues of varying levels of pigmentation.

The tissues are produced using serum free medium without artificial stimulators of melanogenesis such as TPA and IBMX. The cultures are grown on cell culture inserts at the air-liquid interface, allowing for topical application of skin lighteners or self-tanning agents. Thus, the model provides a useful *in vitro* means to evaluate cosmetic and pharmaceutical agents designed [85].

We tested various concentrations of the polyunsaturated fatty acids linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid in the MelanoDerm™ system using ethanol and DMSO as vehicle for application. Our results show that none of the substances has an influence on pigmentation of the MelanoDerm™ tissues.

Taking together the results from the MelanoDerm system and the monolayer system it is obvious that the polyunsaturated fatty acids tested do not affect melanogenesis of human melanocytes *in vitro*. These results suggest that polyunsaturated fatty acids do not influence human pigmentation *in vivo*.

4.3 Concluding remarks

The definitive functions of polyunsaturated fatty acids on melanogenesis still remain unclear. In this work we could demonstrate that linoleic acid, arachidonic acid, α-linolenic acid and γ-linolenic acid do affect the biosynthesis of melanin pigment of murine melanoma cells *in vitro,* whereas in human melanocytes *in vitro* they show no obvious effect on pigmentation.

In the murine model system the polyunsaturated fatty acids seem to have two distinct effects on melanogenesis: the acceleration of secretion of intracellularly produced melanin to the extracellular space and the reduction of intracellular melanin content.

In our opinion these effects are not directly correlated. To prove this hypothesis, the following experimental approaches are possible:

1. Measuring the potential of polyunsaturated fatty acids to cause secretion of intracellularly produced melanin to the extracellular space in a cell culture system consisting of B16 mouse melanoma cells and culture conditions where melanin is not secreted to the surrounding culture fluid. These studies are possible when B16 cells are cultured in a serum free medium and melanogenesis is induced with either melanocyte-stimulating hormone or dibutyryl cAMP. Another interesting question is, if the intracellular melanin content of B16 cells cultured under such conditions is reduced by treatment with polyunsaturated fatty acids.

2. Detection of extracellular tyrosinase activity and enzyme level of cultures of B16 mouse melanoma cells under normal conditions and after treatment with polyunsaturated fatty acids. This will elucidate the question whether darkening of medium is, at least partially, caused by production of dopachrome from tyrosine abundant in the medium.

Although our results show that polyunsaturated fatty acids have no influence on melanogenesis of human melanocytes *in vitro* the hypothesis that polyunsaturated fatty acids do not affect human pigmentation has to be proven by an *in vivo* study.
It remains unclear why polyunsaturated fatty acids affect the pigmentation of murine melanoma cells but not that of human melanocytes. The main conclusion from our work is the fact that melanin modulating agents differ in their actions on melanogenesis when tested in different *in vitro* cell culture models.

5. References

1. Orlow, S.J., *Melanosomes are specialized members of the lysosomal lineage of organelles.* The Journal Of Investigative Dermatology, 1995. **105**(1): p. 3-7.

2. Bahadoran, P., et al., *Rab27a: a key to melanosome transport in human melanocytes.* The Journal of Cell Biology, 2001. **152**(4): p. 843-849.

3. Seiberg, M., *Keratinocyte-melanocyte interactions during melanosome transfer.* Pigment Cell Research, 2001. **14**: p. 236-242.

4. Seiberg, M., et al., *The protease-activated receptor 2 regulates pigmentation via keratinocyte-melanocyte interactions.* Exp. Cell. Res., 2000. **254**: p. 25-32.

5. Kobayashi, N., et al., *Supranuclear melanin caps reduce ultraviolet induced photoproducts in human epidermis.* The Journal Of Investigative Dermatology, 1998. **110**(5): p. 806-810.

6. Jimbow, K. and S. Sugiyama, *Melanosomal translocation and transfer*, in *The Pigmentary System: Physiology and Pathophysiology*, J.J. Nordlund, et al., Editors. 1998, Oxford University Press.

7. Ito, S., K. Wakamatsu, and H. Ozeki, *Chemical analysis of melanins and its application to the study of the regulation of melanogenesis.* Pigment Cell Research, 2000. **13**: p. 103-109.

8. Hearing, V.J. *Biochemical control of melanogenesis and melanosomal organization*. in *JID Symposium*. 1999.

9. Barsh, G.S., *Pigmentation, pleiotropy and genetic pathways in humans and mice.* Am. J. Hum. Gen., 1995. **57**: p. 743-747.

10. Barsh, G.S., *The genetics of pigmentation: from fancy genes to complex traits.* Trends Genet., 1996. **12**: p. 299-305.

11. Valverde, P., et al., *Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans.* Nat. Genet., 1995. **11**: p. 328-330.

12. Thody, A.J., *Epidermal melanocytes: their regulation and role in skin pigmentation.* Eur. J. Dermatol., 1995(5): p. 558-565.

13. Körner, A.M. and J.W. Pawelek, *Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin.* Science, 1982. **217**: p. 1163.

14. Hearing, V.J., et al., *Mammalian tyrosinase: isolation by a simple new procedure and characterization of its steric requirements for cofactor activity.* Arch. Biochem. Biophys., 1978. **185**: p. 407-418.

15. Shibahara, S., et al., *Molecular basis for the heterogeneity of human tyrosinase.* Tohoku. J. Exp. Med., 1988. **156**: p. 403-414.

16. Shibahara, S., et al., *Regulation of pigment cell-specific gene expression by MITF.* Pigment Cell Research, 2000. **13**(Suppl. 8): p. 98-102.

17. Bertolotto, C., et al., *Regulation of tyrosinase gene expression by cAMP in B16 melanoma cells involves two CATGTG motifs surounding the TATA box: implication of the microphthalmia gene product.* J. Cell. Biol., 1996. **134**: p. 747-755.

18. Park, H.Y. and B.A. Gilchrest, *Signaling pathways mediating melanogenesis.* Cellular and Molecular Biology, 1999. **45**(7): p. 919-930.

19. Park, H.Y., et al., *The beta isoform of proteinkinase C stimulates human melanogenesis by activating tyrosinase in pigment cells.* J. biol. Chem., 1993. **268**: p. 11742-11749.

20. Park, H.Y., et al., *Protein kinase C-β activates tyrosinase by phosphorylating serine residues in its cytoplasmatic domain.* J. biol. Chem., 1999. **274**: p. 16470-16478.

21. Jiménez, M., K. Tsukamoto, and V.J. Hearing, *Tyrosinases from two different loci are expressed by normal and by transformed melanocytes.* J. biol. Chem., 1991. **266**: p. 1147-1156.

22. Jackson, I., *A cDNA encoding tyrosinase-related protein maps to the brown locus in mice.* Proc. Natl. Acad. Sci. U. S. A., 1988. **85**(4392-4396).

23. Zhao, N., J. Nordlund, and R.E. Boissy, *Human TRP-1 has tyrosine hydroxylase but no DOPA oxidase activity.* Pigment Cell Research, 1993. **7**: p. 131-140.

24. Jimenez-Cervantes, C., et al., *Tyrosinase isoenzymes in mammalian melanocytes. 1. Biochemical characterization of two melanosomal tyrosinases from B16 mouse melanoma.* Eur. J. Biochem., 1993. **217**: p. 549-556.

25. Halaban, R. and G.E. Moellmann, *Murine and human b locus pigmentation genes encode a glycoprotein (gp75) with catalase activity.* Proc. natn. Acad. Sci., 1990. **87**: p. 4809-4813.

26. Kobayashi, N., et al., *Tyrosinase stabilization by TRP-1 (the brown locus protein).* J. biol. Chem., 1998. **273**: p. 31801-31805.

27. Yokoyama, K., et al., *Cloning the human dopachrome tautomerase/tyrosinase related protein 2 gene and identification of two regulatory regions required for its pigment cell-specific expression.* J. biol. Chem., 1994. **289**: p. 27080-27087.

28. Albino, A.P., B.M. Davis, and D.M. Nanus, *Induction of growth factor RNA expression in human malignant melanoma: markers of transformation.* Cancer Res., 1991. **51**: p. 4815-4820.

29. Tsatmali, M., et al., *Skin POMC peptides: their actions at the human MC-1 receptor and roles in the tanning response.* Pigment Cell Research, 2000. **13**(Suppl. 8): p. 125-129.

30. Hara, M., M. Yaar, and B.A. Gilchrest, *Endothelin-1 of keratinocyte origin is a mediator of melanocyte dendricity.* The Journal Of Investigative Dermatology, 1995. **105**(6): p. 744-748.

31. Yada, Y., K. Higuchi, and G. Imokawa, *Effects of endothelins on signal transduction and proliferation in human melanocytes.* The Journal of Biological Chemistry, 1991. **266**(27): p. 18352-18357.

32. Imokawa, G., et al., *The role of Endothelin-1 in epidermal hyperpigmentation and signaling mechanisms of mitogenesis and melanogenesis.* Pigment Cell Research, 1997. **10**: p. 218-228.

33. Reid, K., et al., *Multiple roles for endothelin in melanocyte development: regulation of progenitor number and stimulation of differentiation.* Development, 1996. **122**: p. 3911-3919.

34. Thody, A.J., et al., *MSH peptides are present in mammalian skin.* Peptides, 1983. **4**: p. 813-816.

35. Wakamatsu, K., et al., *Characterisation of ACTH peptides in human skin and their activation of the melanocortin-1 receptor.* Pigment Cell Research, 1997. **10**: p. 288-297.

36. Lerner, A.B. and J.S. McGuire, *Effect of α- and β-melanocyte stimulating hormones on the skin color of man.* Nature, 1961. **189**: p. 176-179.

37. Friedman, P.S., et al., *α-MSH causes a small rise in cAMP but has no effect on basal or ultraviolet-stimulated melanogenesis in human melanocytes grown in culture.* Br. J. Dermatol., 1990. **123**: p. 145-151.

38. Hunt, G., et al., *α-MSH and its analogue $Nle^4DPhe^7\alpha$-MSH increases the eumelanin: phaeomelanin ration in cultured human melanocytes.* J. Invest. Dermatol., 1994. **104**: p. 83-85.

39. Donatien, P., et al., *The expression of functional MSH receptors in cultured human melanocytes.* Arch. Dermatol. Res., 1992. **284**: p. 424-426.

40. Hunt, G., et al., *Cultured human melanocytes respond to MSH peptides and ACTH.* Pigment Cell Research, 1994. **7**: p. 217-221.

41. Kippenberger, S., et al., *Quantification of tyrosinase, TRP-1, and TRP-2 transcripts in human melanocytes by reverse transcriptase-competitive multiplex PCR - regulation by steroid hormones.* The Journal Of Investigative Dermatology, 1998. **110**(4): p. 364-367.

42. Halaban, R., et al., *Growth factors, receptor kinases, and protein tyrosine phosphatases in normal and malignant melanocytes.* J. Immunother., 1992. **12**: p. 154-161.

43. Pathak, M.A. and K. Stratton, *Free radicals in human skin before and after exposure to light.* Arch. Biochem. Biophys., 1968. **123**: p. 468-476.

44. McGregor, J.M. and J.L.M. Hawk, *Acute effects of ultraviolet radiation on the skin.*, in *Dermatology in general medicine*, I.M. Freedberg, et al., Editors. 1999, McGraw-Hill Inc.: New York. p. 1555-1561.

45. Friedman, P.S. and B.A. Gilchrest, *Ultraviolet radiation directly induces pigment production by cultured human melanocytes.* Cell. Phys., 1987. **133**: p. 88-94.

46. Gilchrest, B.A. and M.S. Eller, *DNA photodamage stimulates melanogenesis and other photoprotective responses.* J. Invest. Dermatol. Symp. Proc., 1999. **4**: p. 35-40.

47. Vishwanath, M.S., *The essential fatty acids.* Nutrition in clinical practice, 1992. **7**: p. 179-186.

48. Burr, G.O. and M.M. Burr, *A new deficiency disease produced by the rigid exclusion of fat from diet.* J. biol. Chem., 1929. **82**: p. 345-367.

49. Burr, G.O. and M.M. Burr, *On the nature of the fatty acids essential in nutrition.* J. biol. Chem., 1930. **86**: p. 587-621.

50. Brown, W.R., A.E. Hansen, and G.O. Burr, *Effects of prolonged use of extremely low-fat diet on an adult human subject.* J. Nutr., 1938. **16**: p. 511-524.

51. Hansen, H.S. and B. Jensen, *Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier. Evidence from feeding studies with oleate, linoleate, arachidonate, columbinate and α-linolate.* Biochem. Biophys. Acta., 1985. **834**: p. 357-363.

52. Ziboh, V.A., C.C. Miller, and Y. Cho, *Metabolism of polyunsaturated fatty acids by skin epidermal enzymes: generation of antiinflammatory and antiproliferative metabolites.* Am. J. Clin. Nutr., 2000. **71**: p. 361-366.

53. Cho, Y. and V.A. Ziboh, *Incorporation of 13-hydroxyoctadeecadienoic acid (13-HODE) into epidermal ceramides and phospholipids: phospholipase C-catalyzed release of novel 13-HODE-containing diacylglycerol.* J. Lipid Res., 1994. **35**: p. 255-262.

54. Ziboh, V.A., *The significance of polyunsaturated fatty acids in cutaneous biology.* Lipids, 1996. **31**: p. 249-253.

55. Fogh, K., et al., *Improvement of psoriasis vulgaris after intralesional injections of 15-hydroxyeicosatetraenoic acid (15-HETE).* J. Am. Acad. Dermatol., 1988. **18**: p. 279-285.

56. Vanderhoek, J.Y., R.V. Bryant, and J.M. Bailey, *Inhibition of leukotriene biosynthesis by the leukocyte product 15-hydroxy-5,5,11,13-eicosatetraenoic acid.* J. biol. Chem., 1980. **225**: p. 11064-10066.

57. Shono, S. and K. Toda, *The effect of fatty acids on tyrosinase activity*, in *Pigment cell*, M. Seiji, Editor. 1982, University of Tokyo Press: Tokyo. p. 263-268.

58. Ando, H., et al., *Linoleic acid and α-linolenic acid lightens ultraviolet-induced hyperpigmentation of the skin.* Arch. Dermatol. Res., 1998. **290**: p. 375-381.

59. Ando, H., et al., *Protein kinase C and linoleic acid-induced inhibition of melanogenesis.* Pigment Cell Research, 1990. **3**: p. 200-206.

60. Ando, H., et al., *Correlation between the number of melanosomes tyrosinase mRNA levels and tyrosinase activity in cultured murine melanoma cells in response to various melanogenesis regulatory agents.* Journal Of Cellular Physiology, 1995. **163**: p. 608-614.

61. Ando, H., et al., *Possible involvement of proteolytic degradation of tyrosinase in the regulatory effect of fatty acids on melanogenesis.* Journal of Lipid Research, 1999. **40**: p. 1312-1316.

62. Eisinger, M. and O. Marko, *Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin.* Proc. Natl. Acad. Si., 1982. **79**: p. 2018-2022.

63. Halaban, R. and F.D. Alfano, *Selective elimination of fibroblasts from cultures of normal human melanocytes.* In Vitro, 1984. **20**: p. 447-450.

64. Borenfreund, E. and J.A. Puerner, *A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR90).* Journal of tissue culture methods, 1985. **9**(1): p. 7-9.

65. Nakajima, M., et al., *κ-Casein suppresses melanogenesis in cultured pigment cells.* Pigment Cell Research, 1996. **9**: p. 235-239.

66. Bhatnagar, V., A. Srirangam, and R. Abburi, *In vitro modulation of proliferation and melanization of melanoma cells by citrate.* Molecular and Cellular Biochemistry, 1998. **187**: p. 57-65.

67. Winder, A.J. and H. Harris, *New assays for the tyrosine hydroxylase and dopa oxidase activities of tyrosinase.* Eur. J. Biochem., 1991. **198**: p. 317-326.

68. Crowther, J.R., *ELISA Theory and Practice*. Methods in molecular biology, ed. J.M. Walker. Vol. 42. 1995, New Jersey: Humana Press.

69. Holland, P.M., et al., *Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase.* Proc. Natl. Acad. Sci. U S A, 1991. **88**: p. 7276-7280.

70. *ABI PRISM 7700 Sequence Detection System User Bulletin 2*. 1997, PE Applied Biosystems.

71. Ito, A., et al., *Glucocorticoid stimulates melanogenesis and tyrosinase gene expression in B16 melanoma cells.* Pigment Cell Research, 1991. **4**: p. 247-251.

72. Park, H.Y. and B.A. Gilchrest, *Reduction of melanogenic activity and responsiveness to α-melanocyte-stimulating hormone during serial passage of melanoma cells.* Journal of Cutaneous medicine and Surgery, 1996. **1**(1): p. 4-9.

73. Laskin, J.D., et al., *Control of melanin synthesis and secretion by B16/C3 melanoma cells.* J. Cell. Phys., 1982. **113**(3): p. 481-486.

74. Buffey, J.A., M. Edgecombe, and S. Mac Neil, *Calcium plays a complex role in the regulation of melanogenesis in murine B16 melanoma cells.* Pigment Cell Research, 1993. **6**(6): p. 385-393.

75. Newman, M.J., *Inhibition of carcinoma and melanoma cell growth by type 1 transforming growth factor β is dependent on the presence of polyunsaturated fatty acids.* Proc. Natl. Acad. Sci., 1990. **87**: p. 5543-5547.

76. Maeda, K., et al., *Phospholipases induce melanogenesis in organ-cultured skin.* Photochemistry and Photobiology, 1996. **64**(1): p. 220-223.

77. Morelli, J.G., et al., *Leukotrienes C4 and D4 as potent mitogens for cultured human neonatal melanocytes.* J Invest Dermatol, 1989. **93**(6): p. 719-722.

78. Fernstrom, J.D., *Effects of dietary polyunsaturated fatty acids on neuronal function.* Lipids, 1999. **34**(2): p. 161-169.

79. Youdim, K.A., A. Martin, and J.A. Joseph, *Essential fatty acids and the brain: possible health implications.* Int. J. Dev. Neurosci., 2000. **18**: p. 383-399.

80. Valverde, P., et al., *Proteolysis with trypsin of mammalian tyrosinase isoforms from B16 mouse melanoma.* Arch. Biochem. Biophys., 1992. **297**(2): p. 221-227.

81. Laskin, J.D. and L.A. Piccinini, *Tyrosinase isozyme heterogeneity in differentiating B16/C3 melanoma.* J. Biol. Chem., 1986. **261**(35): p. 16226-16235.

82. Hearing, V.J., *The melanosome: the perfect model for cellular responses to the environment.* Pigment Cell Research, 2000. **13**(8).

83. Halaban, R., et al., *Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells.* Proc. Natl. Acad. Sci. U S A, 1997. **94**(12): p. 6210-6215.

84. Halaban, R., *The regulation of normal melanocyte proliferation.* Pigment Cell Research, 2000. **13**(1): p. 4-14.

85. Klausner, M., et al., *Use of MelanoDerm™, an epidermal model containing functional melanocytes, for skin lightening studies.* Journal of Investigative Dermatology, 2000. **114**: p. 859.

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7. Curriculum vitae

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