CHARPTER II

LITERATURE REVIEWS

Tamarind (*Tamarindus indica* L.)

Family: Leguminosae (Fabaceae)

Common Names: Tamarind, Tamarindo, Tamarin, Sampalok.

Distant affinity: Carob (Ceratonia siliqua).

Origin: Tamarind is native to tropical Africa and grows wild throughout Sudan. It was introduced into India so long ago and today is widely grown in Thailand. It has often been reported as indigenous there too. It is extensively cultivated in tropical areas of the world. In Thailand, two types of tamarind are found in abundance, the so-called sweet and sour varieties.

Adaptation: Tamarind is well adapted to semiarid tropical condition, although it does well in many humid tropical areas of the world with seasonally high rainfall. Young trees are very susceptible to frost, but mature trees will withstand brief periods of -2°C without serious injury. A tamarind tree in the Quail Botanical Gardens in San Diego County flowers, but rarely sets fruit, possibly because of the cool coastal climate. Dry weather is important during the period of fruit development. The tree is too large to be grown in a container for any length of time.

Growth Habit: Tamarinds are slow-growing, long-lived, evergreen trees that under optimum conditions can grow 80 feet high with a spread of 20 to 35 ft., in its native eastern Africa and Asia. However, in Southern California it seldom reaches more than 15 to 25 ft. in height.

Foliage: The bright green, pinnate foliage is dense and feathery in appearance, making an attractive shade tree with an open branch structure. The leaves are normally evergreen but may be shed briefly in very dry areas during the hot season. There are usually as many as 10 to 20 nearly sessile 1/2 - 1 inch, pale green leaflets per leaf. The leaflets close up at night.

Flowers: The inconspicuous, inch-wide, five-petalled flowers are borne in small racemes and are yellow with orange or red streaks. The flower buds are pink due to the outer color of the 4 sepals, which are shed when the flower opens.

Fruit: The 3 - 8 inch long, brown, irregularly curved pods as shown in Figure 1 are borne in abundance along the new branches. As the pods mature, they fill out somewhat and the juicy, acidulous pulp turns brown or reddish-brown. When fully ripe, the shells are brittle and easily broken. The pulp dehydrates to a sticky paste enclosed by a few coarse stands of fiber. The pods may contain from 1 to 12 large, flat, glossy brown, obovate seeds embedded in the brown, edible pulp. The pulp has a pleasing sweet/sour flavor and is high in both acid and sugar. There are wide differences in fruit size and flavor in seedling trees. Indian types have longer pods with 6 - 12 seeds, while the West Indian types have shorter pods containing only 3 - 6 seeds. Most tamarinds in the Americas are of the shorter type.

Figure 1 Tamarind tree, fruit and seed

Every part of the tamarind tree is useful, especially the fruit. The sweetish acidic pulp of the fruit is a product of commercial importance. It is used in culinary preparations, beverages, like wine and also in cosmetic products. The tender leaves, seeds and flowers are used as vegetables. Interestingly, tamarind seed coat that always be wasted, appear to be important sources of potential antioxidant natural products. The procyanidin profiles detected in tamarind seed coat are similar to those of grapes (Saito, et al., 1998; Castillo, et al., 2000; Peng, et al., 2001), pinto beans and plums (Gu, et al., 2003) and may play a role in health care by acting as cancer chemopreventive agents (Zhao, et al., 1999; Agarwal, et al., 2002; Kozikowski, et al., 2003) and reducing risk of cardiovascular disease (Facino, et al., 1999; Shi, et al., 2003; Berti, et al., 2003). The active compounds that found in tamarind seed coat are shown in Figures 2 and 3.

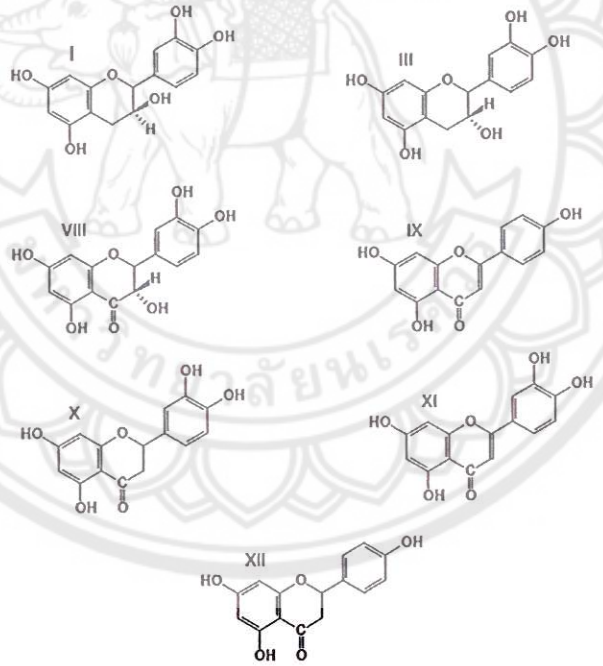

Figure 2 The structures of the monomeric flavonoids including (+)-catechin (I), (-)-epicatechin (III), taxifolin (VIII), apigenin (IX), eriodictyol (X), luteolin (XI) and naringenin (XII) which are found in tamarind seed coat extract

Source: Sudjaroen, et al., 2005

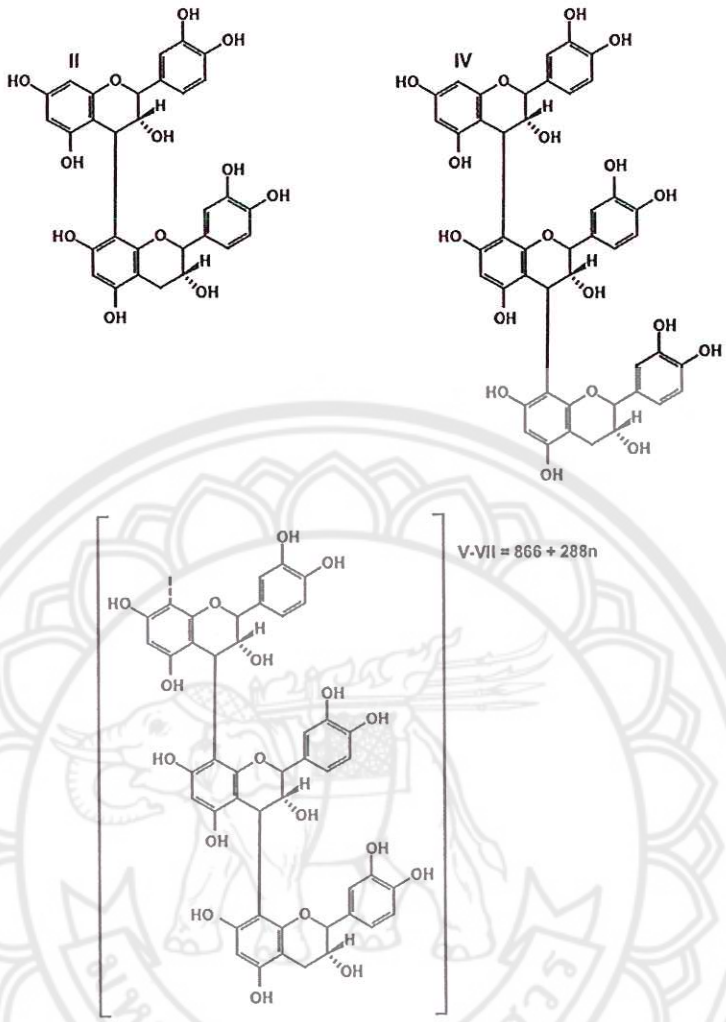

Figure 3 The structures of the oligomeric flavonoids including procyanidin B2 (II), procyanidin trimer (IV), procyanidin tetramer (V), procyanidin pentamer (VI) and procyanidin hexamer (VII) which are found in tamarind seed coat extract

Source: Sudjaroen, et al., 2005

Antioxidant activities of phenolic compound

Most natural products can be classified into three major groups: terpenoids, alkaloids, and phenolic compounds (mostly phenylpropanoids). Terpenoids are composed of five-carbon units synthesized by way of the acetate/mevalonate pathway or the glyceraldehyde 3-phosphate/pyruvate pathways. Many plant terpenoids are toxins and feeding deterrents to herbivores or are attractants of various sorts. Alkaloids are synthesized principally from amino acids. These nitrogen-containing compounds protect plants from a variety of herbivorous animals, and many possess pharmacologically important activity. Phenolic compounds, which are synthesized primarily from products of the shikimic acid pathway, have several important roles in plants. Tannins, lignans, flavonoids, and some simple phenolic compounds serve as defenses against herbivores and pathogens phenolics are a class of chemical compounds consisting of a hydroxyl group attached to an aromatic hydrocarbon group. The simplest of the class is phenol. Although similar to alcohols, phenols have unique properties and are not classified as alcohols (since the hydroxyl group is not bonded to a saturated carbon atom). They have relatively higher acidities due to the aromatic ring's tight coupling with the oxygen and a relatively loose bond between the oxygen and hydrogen. The acidity of the hydroxyl group in phenols is commonly intermediate between that of aliphatic alcohols and carboxylic acids (their pK_a is usually between 10 and 12). Loss of a positive hydrogen ion (H^+) from the hydroxyl group of a phenol forms a negative phenolate ion.

The free radical scavenging properties of flavonoids have permitted characterization of the major phenolic components of naturally occurring phytochemicals as antioxidants. The flavonoids are a large class of compounds, ubiquitous in plants, and usually occurring as glycosides. They contain several phenolic hydroxyl functions attached to ring structures, designated A, B and C (Figure 4). Structural variations within the rings subdivide the flavonoids into several families:

1. Flavonols (e.g. quercetin and kaempferol), with the 3-hydroxy pyran-4-one C ring.

2. Flavones (e.g. luteolin, apigenin and chrysin), lacking the 3-hydroxyl group.

3. Flavanols (e.g. catechin), lacking the 2,3-double bond and the 4-one structure.

4. Isoflavones (e.g. genistein), in which the B ring is located in the 3 position on the C ring.

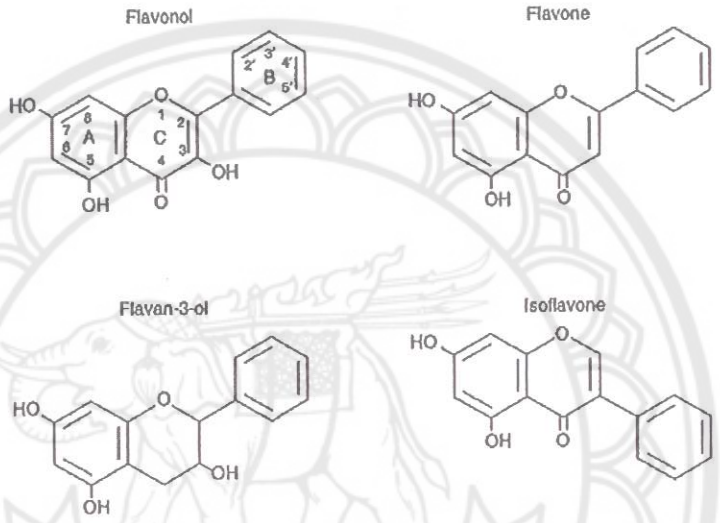

Figure 4 Structures of the flavonoids. The basic structure consists of the fused A and C rings, with the phenyl B ring attached through its 1' position to the 2-position of the C ring (numbered from the pyran oxygen). Types shown include: flavonols (3 hydroxyflavones) [e.g. quercetin (3,5,7,3',4'-hydroxyl) and kaempferol (3,5,7,4'- hydroxyl)]; flavones [e.g. luteolin (5,7,3',4'-hydroxyl), apigenin (5,7,4'-hydroxyl) and chrysin (5,7,-hydroxyl)]; flavan-3-ols [e.g. catechin (3,5,7,3',4'-hydroxyl)]; and isoflavones [e.g. genistein (5,7,4'-hydroxyl)]

Source: Cos, 2003

Relationships between the structure and antioxidant activity of phenols

The chemical activities of polyphenols in terms of their reducing properties as hydrogen or electron-donating agents predicts their potential for action as free-radical scavengers (antioxidants). The activity of an antioxidant is determined by:

1. Its reactivity as a hydrogen or electron-donating agent (which relates to its reduction potential).

2. The fate of the resulting antioxidant-derived radical, which is governed by its ability to stabilize and delocalize the unpaired electron.

3. Its reactivity with other antioxidants.

4. The transition metal-chelating potential.

Polyphenols possess ideal structural chemistry for free radical-scavenging activities, and have been shown to be more effective antioxidants *in vitro* than vitamins E and C. In addition, the propensity for metal chelation, particularly iron and copper, supports the role of polyphenols as preventative antioxidants in terms of inhibiting transition metal catalysed free radical formation. The structural arrangements imparting greatest antioxidant activity are (Miller and Rice, 1996):

1. The ortho 3',4'-dihydroxy moiety in the B ring (e.g. in catechin, luteolin and quercetin).

2. The meta 5,7-dihydroxy arrangements in the A ring (e.g. in kaempferol, apigenin and chrysin).

3. The 2,3-double bond in combination with both the 4-keto group and the 3-hydroxyl group in the C ring, for electron delocalization (e.g. in quercetin), as long as the o-dihydroxy structure in the B ring is also present. However, alterations in the arrangement of the hydroxyl groups and substitution of contributing hydroxyl groups by glycosylation decreases the antioxidant activity.

4. For metal chelation, the two points of attachment of transition metal ions to the flavonoid molecule are the o-diphenolic groups in the 3',4'-dihydroxy positions in the B ring, and the ketol structures 4-keto, 3- hydroxy or 4-keto and 5- hydroxy in the C ring of the flavonols.

This was rationalized on the basis that both electrochemical oxidation and hydrogen donating free radical-scavenging involve the breaking of the same phenolic bond between oxygen and hydrogen, producing the phenoxyl radical and hydrogen radical, which consists of an electron and hydrogen ion.

The function and structure of the skin

The skin is the interface between humans and their environmental which the largest organ in the body. It acts as a barrier, protecting the body from harsh external conditions and preventing the loss of important body constituents, especially water. The skin has two layers. The outer is epithelial, the epidermis, which is firmly attached to, and supported by connective tissue in the underlying dermis. Beneath the dermis is loose connective tissue, the subcutis/hypodermis which usually contains abundant fat.

Epidermis

The epidermis is formed from many layers of closely packed cells, the most superficial of which are flattened and filled with keratins. The epidermis contains no blood vessels. It varies in thickness from less than 0.1 mm on the eyelids to nearly 1mm on the palms and soles. As dead surface are shed, the thickness is kept constant by cells dividing in the deepest layer. A generated cell moves, or is pushed by underlying mitotic activity, to the surface, passing through the prickle and granular cell layers before dying in the horny layer. The journey from the basal layer to the surface (epidermal turnover or transit time) takes about 60 days. During this time the appearance of the cell changes. A vertical section through the epidermis summarizes the life history of a single epidermal cell.

1. Cells in the epidermis

Keratinocytes make up about 85% of cells in the epidermis, but three other types of cell are also found there: melanocytes, Langerhans cells and Merkel cells (Figure 5).

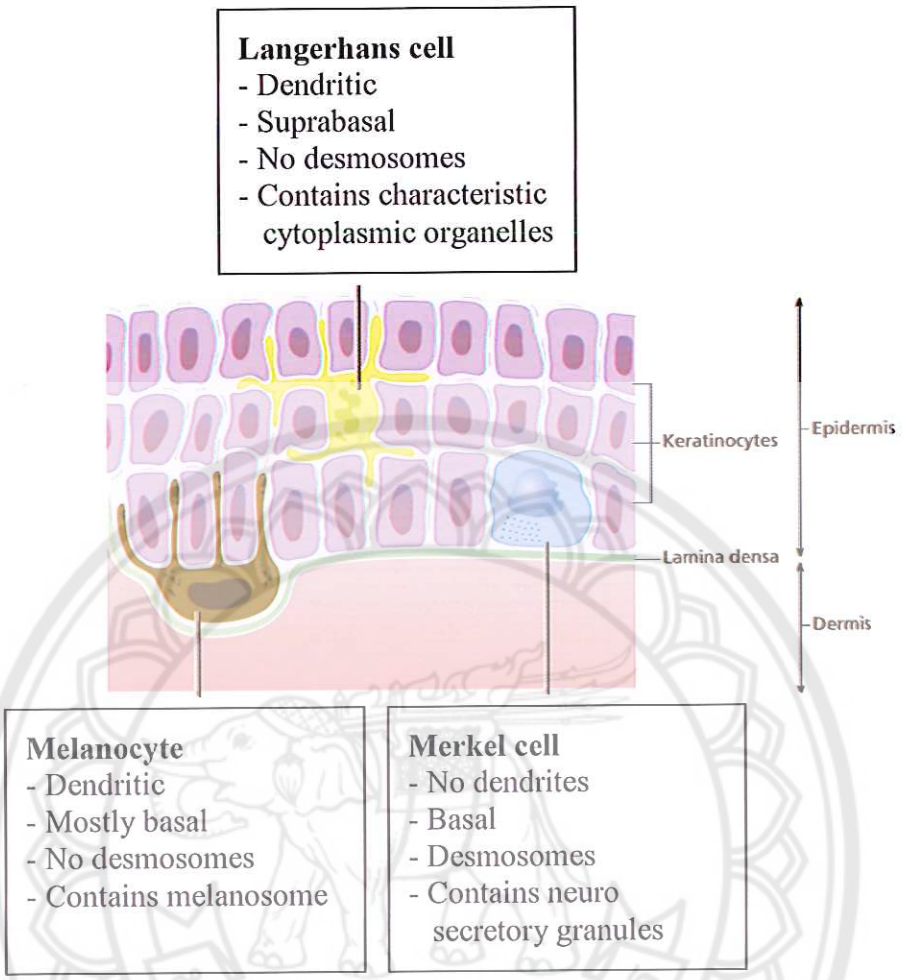

Figure 5 Keratinocyte, Melanocyte, Langerhans cell and Merkel cell in the epidermis

Source: Hunter, 2002

Dermis

The dermis lies between the epidermis and the subcutaneous fat. It supports the epidermis structurally and nutritionally. Its thickness varies, being greatest in the palms and soles and least in the eyelids and penis. In old age, the dermis thins and loses its elasticity. The dermis interdigitates with the epidermis so that upward projections of the dermis, it is important in the adhesion between epidermis and dermis as it increases the area of contact between them.

Like all connective tissues the dermis has three components: cells, fibres and amorphous ground substance.

1. Cells of the dermis

The main cells of the dermis are fibroblasts, but there are also small numbers of resident and transitory mononuclear phagocytes, lymphocytes, Langerhans cells and mast cells. Other blood cells, e.g. polymorphs, are seen during inflammation.

2. Fibres of the dermis

The dermis is largely made up of interwoven fibres, principally of collagen, packed in bundles. Those in the papillary dermis are finer than those in the deeper reticular dermis. When the skin is stretched, collagen, with its high tensile strength, prevents tearing, and the elastic fibres, intermingled with the collagen, later return it to the unstretched state.

Collagen makes up 70–80% of the dry weight of the dermis. Its fibres are composed of thinner fibrils, which are in turn made up of microfibrils built from individual collagen molecules. These molecules consist of three polypeptide chains (molecular weight 150kDa) forming a triple helix with a non-helical segment at both ends. The alignment of the chains is stabilized by covalent cross-links involving lysine and hydroxylysine. Collagen is an unusual protein as it contains a high proportion of proline and hydroxyproline and many glycine residues; the spacing of glycine as every third amino acid is a prerequisite for the formation of a triple helix. There are many, genetically distinct, collagen proteins, all with triple helical molecules, and all rich in hydroxyproline and hydroxylysine. The distribution of some of them is summarized in Table 1.

Table 1 Distribution of some types of collagen

Collagen type	Tissue distribution
I	Most connective tissues including tendon and bone
	Accounts for approximately 85% of skin collagen
II	Cartilage
III	Accounts for about 15% of skin collagen
	Blood vessels
IV	Skin (lamina densa) and basement membranes of other tissues
V	Ubiquitous, including placenta
VII	Skin (anchoring fibrils)
	Fetal membranes

Fibroblast contraction

Extracellular matrix is increasingly being identified as playing a complex and important role in many biological processes. The collagen proteins are a major component of the extracellular matrix in skin tissues and contribute significantly to its structure by forming collagen fibers. Collagen is produced by fibroblasts in the form of procollagen precursors and polymerizes into fibrils, which combine to form a fibrous network or matrix (Alberts, et al., 1994). The procollagen molecules are released via secretory vesicles, which fuse with the cell membrane to create deep, narrow recesses in the fibroblast cell surface. It is in these recesses that the collagen fibrils are formed. Birk and Trelstad, (1986) theorize that these deep recesses give the fibroblast control over the micro-environment in which the collagen fibrils are forming, and thus control over the structure of the collagen matrix. This provides a link between the fibroblast and collagen orientations. Conversely, the collagen matrix is an essential framework, which the fibroblasts use as scaffolding to crawl along. Thus the collagen orientation also influences the orientation of fibroblasts and their ability to move. Among the various biological alignment systems, one that has been extensively modeled is the intracellular actin filament network, which shows pronounced alignment patterns in response to the local stress field.

Form and function of multicellular organisms depends on tissue-specific programs of cell locomotion (Trinkaus, 1984). Much of what is known about cell locomotion comes from studies of cell migration, especially of fibroblast cells, on rigid, planar substrata. The mechanics of how cells exert force necessary for collagen matrix remodeling is unclear. Immediately after polymerization, the collagen matrix is highly pliable and cells remodel the matrix as they begin to spread (Grinnell, 1994; Eastwood, et al., 1996; Freyman, et al., 2002). As remodeling progresses, the overall mechanical properties of the matrix change, which in turn can influence the cells' tractional activity (Brown, et al., 1998; Tranquillo, 1999; Grinnell, 2000; Shreiber, et al., 2001), which involve the myofibroblast differentiation.

In aged skin, fibroblasts lose their capacity to adhere and move over collagen fibers, thereby limiting their ability to reorganize and reorient dermal tissue including collagen fibers (Püschel, et al., 1995; Tamariz, et al., 2002; Jouandeaud, et al., 2004) compared to normal skin (Figure 6). The changes occurring during aging are associated with alterations in skin appearance due to loss of tensile strength with wrinkle formation.

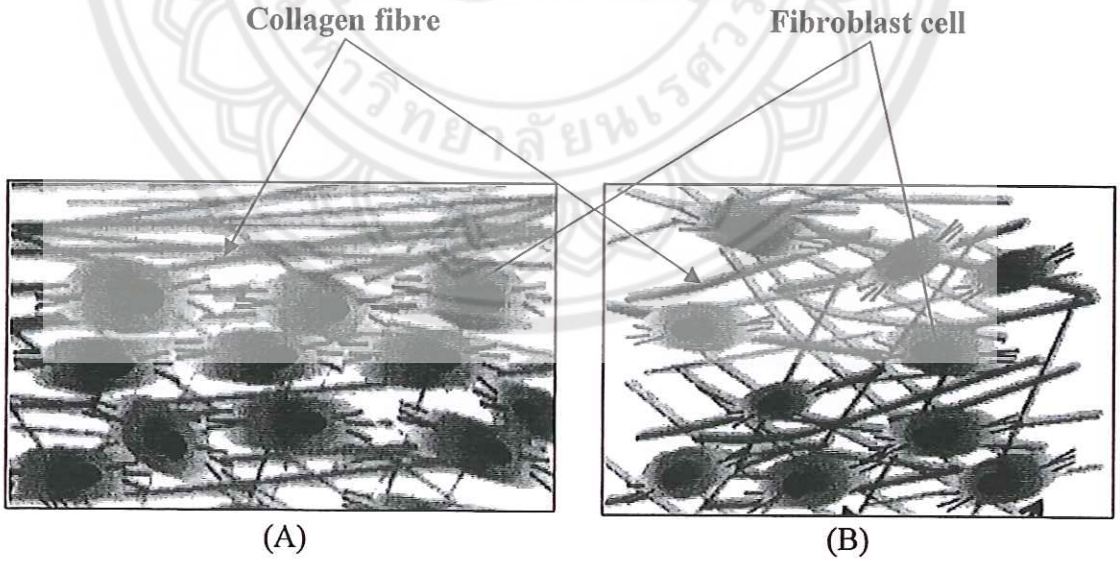

Figure 6 The orientation, locomotion and contraction of fibroblast and collagen in normal skin (A) and aged skin (B)

Definition of the myofibroblast

The myofibroblast has been shown to, firstly, produce mechanical force, by the expression of α-smooth muscle (SM) actin, the actin isoform typical of vascular SM cells and the formation of specialized junctional complexes with the ECM (Dugina, 2001; Goffin, 2006) (Figure 7) and, secondly, synthesize collagen type I and III (Tomasek, 2002); all these changes take place under the stimulation of local mechanical forces and of transforming growth factor (TGF). Thus, the myofibroblast appears as a major player in connective tissue rearrangement and repair. Myofibroblastic differentiation of fibroblastic cells begins with the appearance of the protomyofibroblast, whose stress fibers contain only b- and g-cytoplasmic actins. Protomyofibroblasts evolve, but not necessarily always, into the differentiated myofibroblast, the most common variant of this cell, with stress fibers containing α-SM actin .

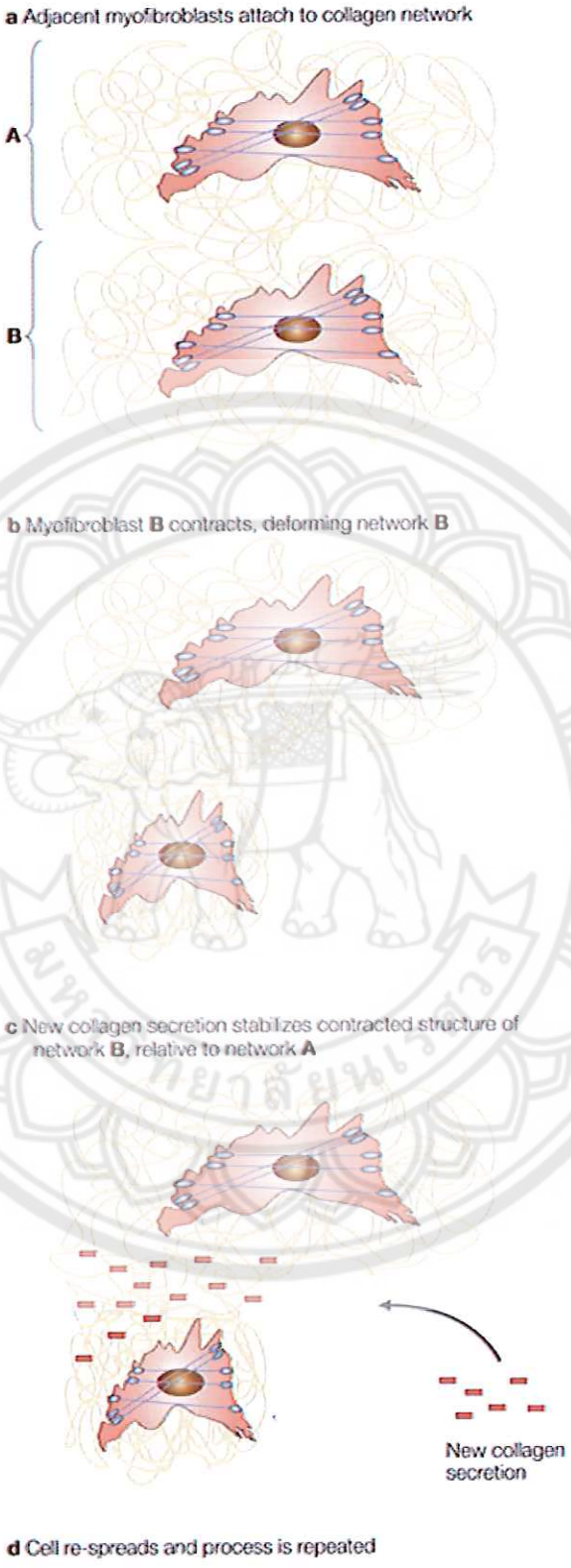

Figure 7 Myofybroblast activities

Source: www.naturereviews.com

Myofibroblasts can, according to the experimental or clinical situation, express other SM cell contractile proteins, such as SM-myosin heavy chains or desmin; however, the presence of α-SM actin represents the most reliable marker of the myofibroblastic phenotype. It has also been shown that α-SM actin is crucial for focal adhesion maturation in myofibroblasts (Hinz, 2003). The modifications of the fibroblast capacities to acquire a myofibroblast phenotype with age are unclear. However, the differentiation of fibroblasts into myofibroblasts with high contractile properties is affected by the age-related dependent fibroblast activation.

Cell Cycle

In normal skin some 30% of basal cells are preparing for division (growth fraction). Following mitosis, a cell enters the G1phase, synthesizes RNA and protein, and grows in size (Figure 8). Later, when the cell is triggered to divide, DNA is synthesized (S phase) and chromosomal DNA is replicated. A short post synthetic (G2) phase of further growth occurs before mitosis (M). DNA synthesis continues through the S and G 2phases, but not during mitosis. The G1 phase is then repeated, and one of the daughter cells moves into the supra- basal layer. It then differentiates, having lost the capacity to divide, and synthesizes keratins. Some basal cells remain inactive in a so-called G0 phase but may re-enter the cycle and resume proliferation. The cell cycle time in normal human skin is controversial; estimates of 50–200 h reflect differing views on the duration of the G phase.

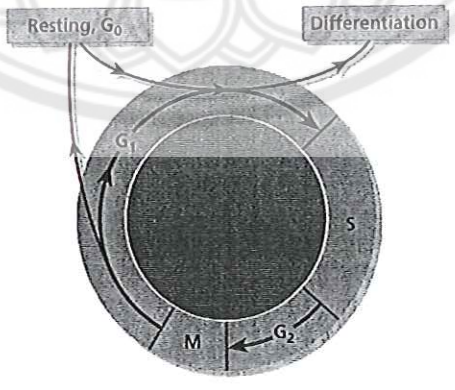

Figure 8 Diagram of cell cycle

Source: Hunter, 2002

Skin reactions to light

Ultraviolet radiation (UVR) is the leading cause of skin aging, cancers, and causes or worsens several skin disorders. UVR is non-ionizing, but changes the skin chemically by reacting with endogenous light-absorbing chemicals (chromophores), which include DNA, RNA, urocanic acid and melanin. The UVR spectrum is divided into three parts (Figure 9), each having different effects on the skin, although UVC does not penetrate the ozone layer of the atmosphere and is therefore currently irrelevant to skin disease. Virtually all of the UVB is absorbed in the epidermis, whereas some 30% of UVA reaches the dermis. The B wavelengths (UVB: 290–320 nm) cause sunburn. The A spectrum (UVA) is long-wave ultraviolet light, from 320 nm to the most violet colour perceptible to the eye (about 400 nm). It ages and tans the skin.

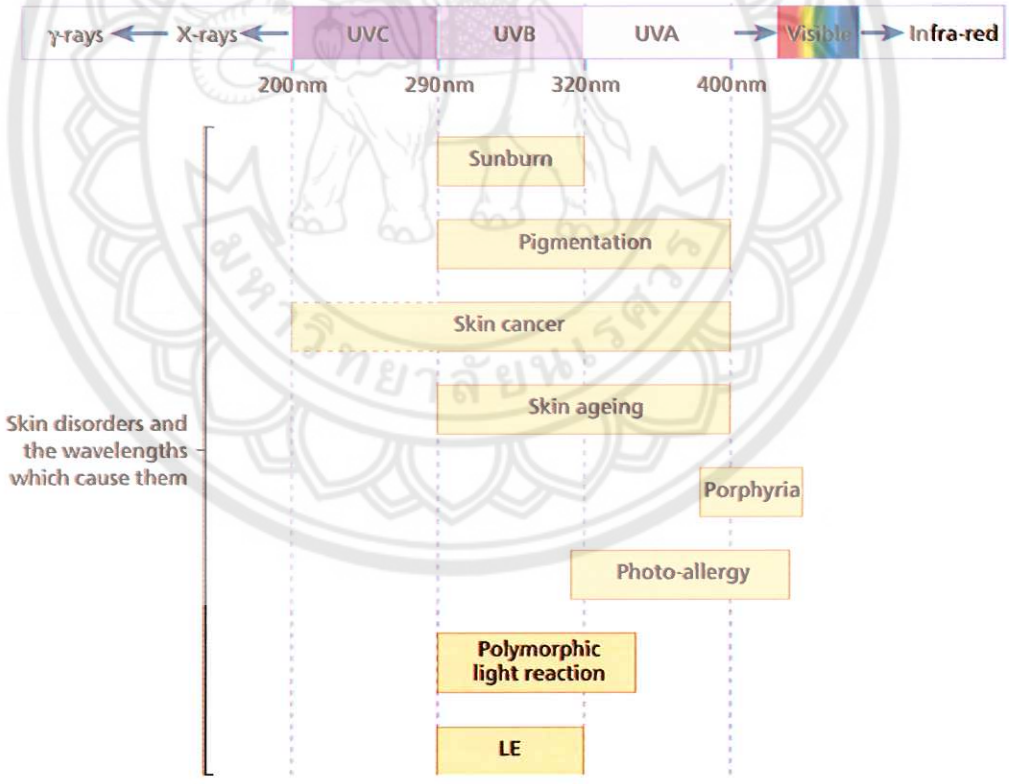

Figure 9 Skin disorders and the wavelengths that cause them

Source: Freinkel R.K., 2001

Skin Pigmentation

Visible pigmentation of the skin, hair, and eyes depends primarily on the functions of melanocytes, a very minor population of cells that specialize in the synthesis and distribution of the pigmented biopolymer melanin. Melanocytes are derived from precursor cells (called melanoblasts) during embryological development, and melanoblasts destined for the skin originate from the neural crest. The accurate migration, distribution, and functioning of melanoblasts/melanocytes determine the visible phenotype of organisms ranging from simple fungi to the most complex animal species. In human skin, melanocytes are localized at the dermal/epidermal border in a characteristic regularly dispersed pattern. Each melanocyte at the basal layer of the epidermis is functionally connected to underlying fibroblasts in the dermis and to keratinocytes in the overlying epidermis. Those three types of cells are highly interactive and communicate with each other via secreted factors and their receptors and via cell/cell contacts to regulate the function and phenotype of the skin.

Melanins: Biochemistry, Biology and Physiology

Melanins are polymorphous and multifunctional biopolymers that are the end products of a multistep biosynthetic chain that is mainly regulated by the activity of tyrosinase and TRPs genes.

Tyrosinase is a copper-containing enzyme that must be folded into a three-dimensional entity to become fully functional. It catalyzes the first steps of the melanogenic pathway: the synthesis of dopaquinone via hydroxylation and oxidation of L-tyrosine or L-DOPA (and alternatively the direct conversion of tyrosine to dopaquinone) and the subsequent oxydation of L-DOPA to form the o-quinone product (Figure 10). TRP1 and TRP2 regulate/stabilize the enzymatic activities of tyrosinase and the eumelanin synthetic rate. In addition, TRP1 improves the structural melanosome integrity.

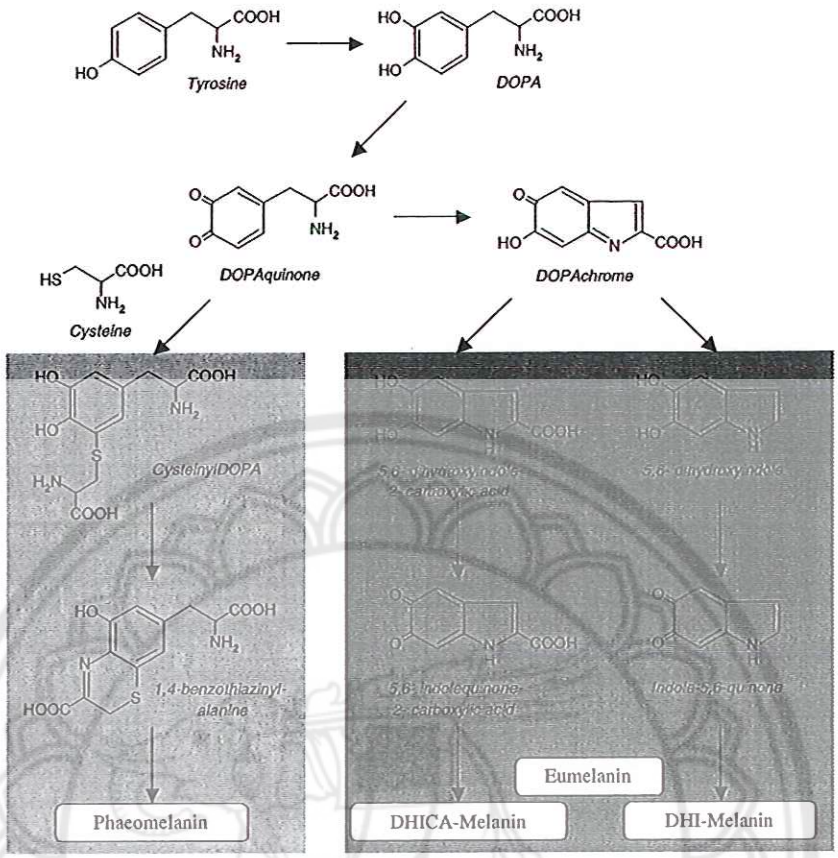

Figure 10 Simplified synthetic pathway for the formation of melanin

Source: Ortel, 2007

Eumelanins behave like polyanions with the capability to reversibly bind cations and polyamines in reactions facilitated by their high carboxyl group content. Its semiquinone units cause a stable paramagnetic state and redox activities with both reducing and oxidizing capabilities towards oxygen radicals and other chemical redox systems. In the phaeomelanogenetic process, dopaquinone is conjugated to cysteine to yield cysteinyldopa that is then metabolized to cysteinyl-DOPA quinone, cyclocysdopaquinonimine and benzothiazinylalanine that is polymerized to phaeomelanin. This reddish-yellow pigment is alkali soluble and has highly variable nitrogen and sulphur contents. Phaeomelanins are photolabile, and their photolysis yields toxic products, such as superoxide, hydroxyl radicals, and hydrogen peroxide.

The absolute cellular amount and the ratio of formation of eu- and phaeo-melanins are not only determined by the enzymatic library but also by the availability to the melanocytes of metal ions (such as manganese, copper, zinc, and iron), cysteine and antioxidant enzymes (such as catalase, superoxide dismutases, peroxidases, glutathione peroxidase, glutathione, reductase, and thior- edoxin reductase/thioredoxin), (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (6-BH4) and phenylalanine and tyrosine hydroxylase (Schallreuter, 1994) .

Stimulators of Melanogenesis

The pigmentary activities of pituitary hormones were first recognized about a century ago, it was demonstrated that adrenocorticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH) are the pituitary hormones responsible of skin darkening in human and hair color changes. Both are efficient in stimulating human pigmentation when they are injected at pharmacologically active doses every day for several weeks.

α-MSH and ACTH are melanocortins (MCs): a structurally related group of peptides that is proteolytically derived from the precursor protein proopiomelanocortin (POMC). This group also includes additional forms of MSH (β-MSH and γ-MSH) as well as lipotrophins and endorphins that do not have melanogenic activities. POMC is synthesized and undergoes proteolytic cleavage at a number of body sites including pituitary gland and epidermal and dermal cell populations. Melanocytes produce POMC and its yield is enhanced following stimulation by another cell population (paracrine mechanism) as well as with a direct ''autocrine'' upregulation of its gene expression, synthesis and processing that can be triggered by ultraviolet radiation (UVR), cytokines, growth factors, and cyclic adenosine monophosphate (cAMP) (Figure 11) (Slominski, 2000). Human melanocytes process POMC with an enzymatic system that has been identified in the melanosomes. Melanogenic activities of MCs follow their specific binding to MC receptors (MCR), a family of A-class rhodopsin-like seven transmembrane spanning G protein coupled receptors (GPCR). Of five known MC receptors (MC1R to MC5R), only melanocortin-1 receptor (MC1R) plays a role in pigmentation. MC1R has an equally high affinity for α-MSH and ACTH. MC binding to MC1R stymulates adenylate cyclase which increases cyclic AMP (cAMP) levels leading to stimulation of tyrosinase and post-DOPA oxidase steps of

melanogenesis. In turn, increased levels of cAMP induce concomitantly enhanced MCR expression. MC1R activation not only regulates the amount of pigment production but it also switches the production of phaeomelanins to that of eumelanins and induces dendrite formation in normal human melanocytes. The MC1R expression in cultured normal human melanocytes is upregulated by α-MSH, endothelin-1 (ET-1), basic fibroblast growth factor (bFGF), and b-estradiol and down-regulated by testosterone and the Agouti signaling protein (Scott, 2002).

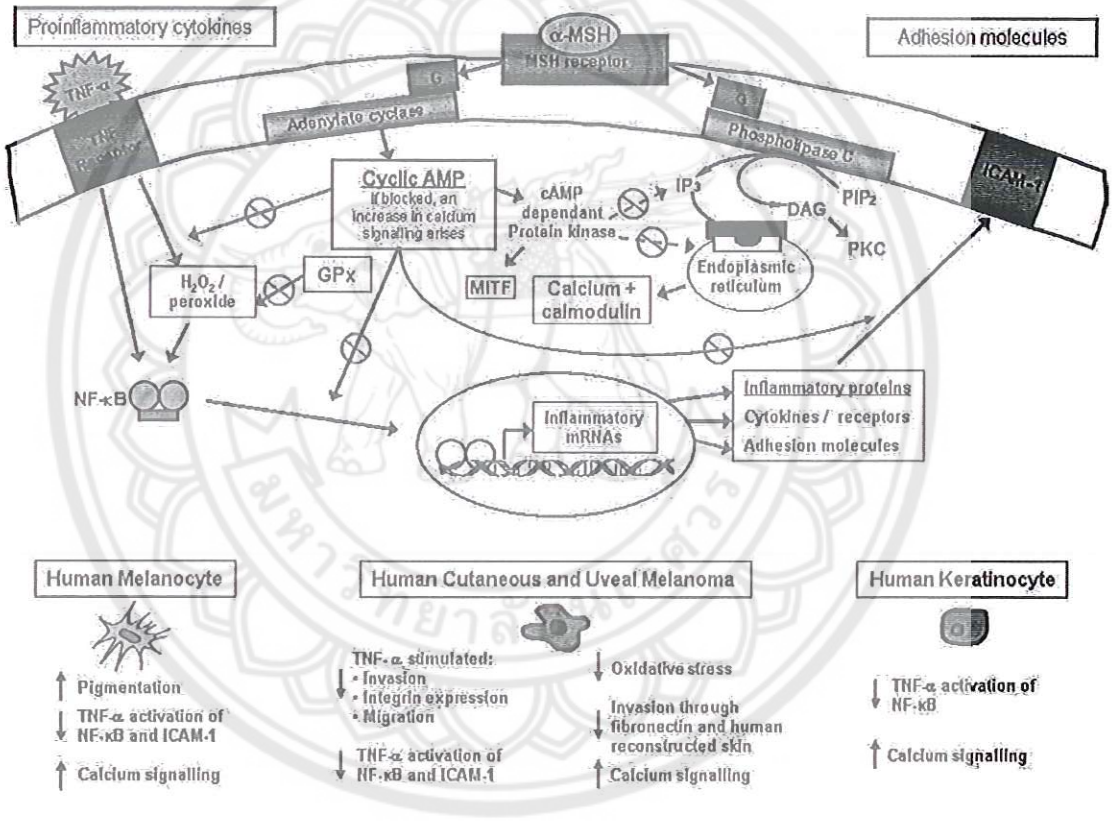

Figure 11 The role of alpha melanocyte stimulating hormone (α-MSH)

Source: Eves, 2006

Neuropeptides also seem to have a strong activity in the control of skin color. Epidermal human melanocytes both in vitro and in vivo have been found to express a b-endorphin/m-opiate receptor system and b-endorphin has potent mitogenic and dendritogenic effects. In addition, it increases the production of melanized pigment granules and facilitates their active transfer to recipient keratinocytes (Kauser, et al., 2003).

Intracellular signals for melanin synthesis

Skin colors in humans range from extremely fair/light to extremely dark depending on racial/ethnic background, but the density of melanocytes in a given area (*e.g.* the back or arms) is virtually identical in all types of skin. Keratinocytes in fair skin tend to cluster their poorly pigmented melanosomes above the nuclei, whereas in dark skin the heavily pigmented melanosomes are distributed individually in keratinocytes, thus maximizing their absorption of light. There is a large intra individual variation in melanocyte density in different areas of the body. Constitutive melanocyte density in the skin can be affected by the environment such as by chronic ultraviolet radiation (UV) (which can increase melanocyte density by 3- or 4-fold) or by toxic compounds such as hydroquinone (which can selectively and permanently destroy melanocytes in the skin). Inherited pigmentary disorders can also result in increased melanocyte density such as freckles or in decreased melanocyte density such as vitiligo. Epidermal melanocytes proliferate slowly, if at all, under normal circumstances, and they are quite resistant to apoptosis because of their high expression of Bcl2. Melanocyte density and differentiation is influenced by the environment, including UV and factors secreted by neighboring keratinocytes and fibroblasts (Figure 12). For example, it was recently shown that fibroblasts in the dermis of the palms/soles secrete high levels of DKK1, which suppresses melanocyte growth and function by inhibiting the Wnt/β-catenin signaling pathway. DKK1 inhibition of Wnt signaling in melanocytes dramatically inhibits the melanogenic pathway, ranging from effects on transcriptional regulators (such as MITF) to downstream melanogenic proteins. DKK1 also affects keratinocytes in overlying epidermis, reducing their uptake of melanin and inducing a thicker less pigmented skin phenotype. The dermis in adult skin retains the expression patterns of HOX genes, which regulate patterning in primary and secondary axes of the embryo, suggesting

that HOX genes regulate site-specific homeostasis even in adult tissue. This finding implies that an upstream regulator of DKK1 may be a specific HOX gene. One major determinant of pigment phenotype of the skin is the melanocortin 1 receptor (MC1R), a G protein-coupled receptor that regulates the quantity and quality of melanins produced. MC1R function is controlled by the agonists α-melanocyte-stimulating hormone (αMSH) and adrenocorticotropic hormone (ACTH) and by an antagonist, Agouti signaling protein (ASP). Activation of the MC1R by an agonist stimulates the expression of the melanogenic cascade and thus the synthesis of eumelanin, whereas ASP can reverse those effects and elicit the production of pheomelanin. αMSH and ACTH can also up-regulate expression of the MC1R gene, thus acting in a positive feedback loop. MC1R function controls the switch to produce eupheomelanin, but the mechanism(s) underlying that switch remains unknown. In sum, constitutive skin pigmentation is determined by:

(*a*) the migration of melanoblasts to that tissue during development,

(*b*) their survival and differentiation to melanocytes,

(*c*) the density of melanocytes,

(*d*) the expression/function of enzymatic and structural constituents of melanosomes

(*e*) the synthesis of different types of melanin (eu- and pheomelanin),

(*f*) the transport of melanosomes to dendrites,

(*g*) the transfer of melanosomes to keratinocytes, and finally

(*h*) the distribution of melanin in suprabasal layers of the skin.

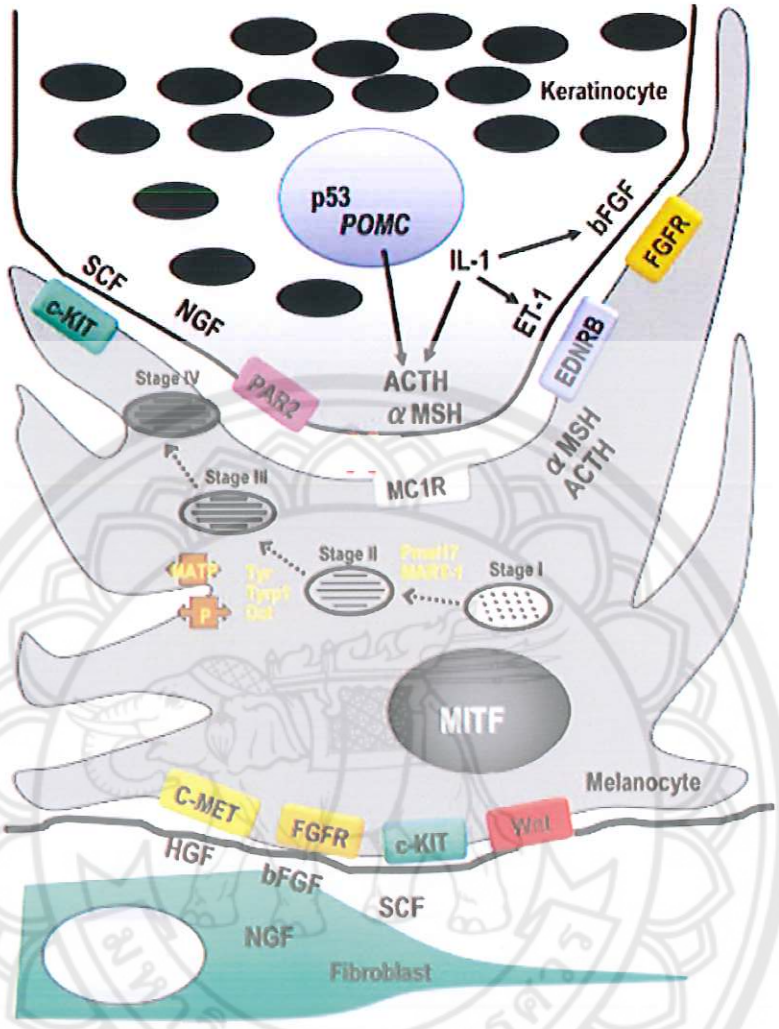

Figure 12 Schematic of receptors, ligands,and other factors that regulate pigmentation of human skin

Source: Yamakuchi, 2007

Protease activated receptor-2 (PAR-2) and pigmentation

Proteases acting at the surface of cells generate and destroy receptor agonists and activate and inactivate receptors, thereby making a vitally important contribution to signal transduction. Certain serine proteases that derive from the circulation (e.g., coagulation factors), inflammatory cells (e.g., mast cell and neutrophil proteases), and from multiple other sources (e.g., epithelial cells, neurons, bacteria, fungi) can cleave protease-activated receptors (PARs) (Figure 13), a family of four G protein-coupled receptors. Cleavage within the extracellular amino terminus exposes a tethered ligand domain, which binds to and activates the receptors to initiate multiple signaling cascades. Despite this irreversible mechanism of activation, signaling by PARs is efficiently terminated by receptor desensitization (receptor phosphorylation and uncoupling from G proteins) and downregulation (receptor degradation by cell-surface and lysosomal proteases). Protease signaling in tissues depends on the generation and release of proteases, availability of cofactors, presence of protease inhibitors, and activation and inactivation of PARs.

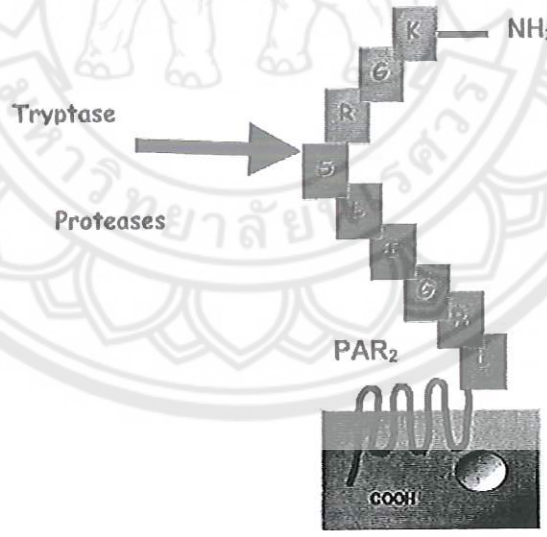

Figure 13 Enzymatic activation mechanism of PAR-2, endogenous protease such as trypsin, tryptase enzymatically cleave the N-terminal sequence of PAR-2 at a specific site (arrow)

Source: www.scielo.br

Cells in the epidermis and dermis could be regulated by proteases of the coagulation cascade. Inflammation of the skin is associated with an influx of inflammatory cells that release proteases that activate PAR-2. Thus whereas in normal skin mast cells are found in the dermis, in atopic dermatitis and psoriasis there is an influx of tryptase-containing mast cells in the dermal and epidermal junction, as well as the epidermis. In addition, epidermal cells express proteases, some of which could activate PARs. Potential activating enzymes include stratum corneum tryptic enzyme and chymotryptic enzyme. However, it remains to be determined if proteases such as these activate PARs. Exogenous proteases from mites, bacteria, and fungi could also signal to epidermal cells.

There is considerable interest in the role of PAR-2 in pigmentation. The interaction between keratinocytes and neighboring melanocytes is essential for skin pigmentation. Melanocytes produce secretory granules, melanosomes, which produce melanin. Melanin-containing melanosomes are transferred to dendrites of melanocytes and are then taken up by keratinocytes by phagocytosis, resulting in skin darkening. Although melanocytes do not express PAR-2, PAR-2 activators regulate the phagocytosis of melanosomes by keratinocytes and thereby control pigmentation. Thus trypsin and PAR-2 AP stimulate pigmentation of cocultures of keratinocytes and melanocytes. Remarkably, the topical application of PAR-2 induces pigmentation of human skin transplanted onto mice. Conversely, a serine protease inhibitor causes lightening of the skin (Seiberg M., 2001). The mechanism of these effects of PAR-2 on pigmentation depends on the stimulation of phagocytosis in keratinocytes. Thus activation of PAR-2 increases the ingestion of particles and bacteria by keratinocytes (Seiberg, 2000). Phagocytosis by keratinocytes not only plays a role in pigmentation but also enables "non- immune" cells such as these to participate in protection of the skin, with implications for wound healing and inflammation. Of particular interest, the increased phagocytic response of keratinocytes correlates with increased activity of soluble serine proteases. Serine protease inhibitors downregulated both constitutive and PAR-2 stimulated phagocytosis, suggesting that these proteases also activate PAR-2 and thereby amplify the response. PAR-2 is upregulated in keratinocytes after ultraviolet exposure, with implications for pigmentation (Scott, 2001).

Sunlight and melanin

Both UVA and UVB wavebands are powerful stimulators of melanogenesis although via different biological mechanisms. The human skin pigmentary response to UV is biphasic with an immediate darkening effect, induced by UVA and visible radiation, and a delayed longer lasting response (tanning), predominantly induced by UVB. The immediate pigment darkening (IPD) is rapid (within minutes), and transient (fades within hours). Persistent pigment darkening (PPD) may be regarded as the portion of the IPD response that remains stable 2 hours post-exposure. There is no evidence that IPD and PPD are photoprotective (Chardon, 1997). It is important to distinguish between IPD/ PPD and true melanogenesis, also known as delayed or ''true'' tanning.

At different times after a single minimally erythemogenic dose of UVR, human skin of various races does not show an increase but rather a decrease of melanocyte density at the lower epidermal levels (Figure 14). The most significant change within the first week after UV exposure appears to be a redistribution of melanin from the lower to the middle layer of the skin, which is more pronounced in the darker skin.

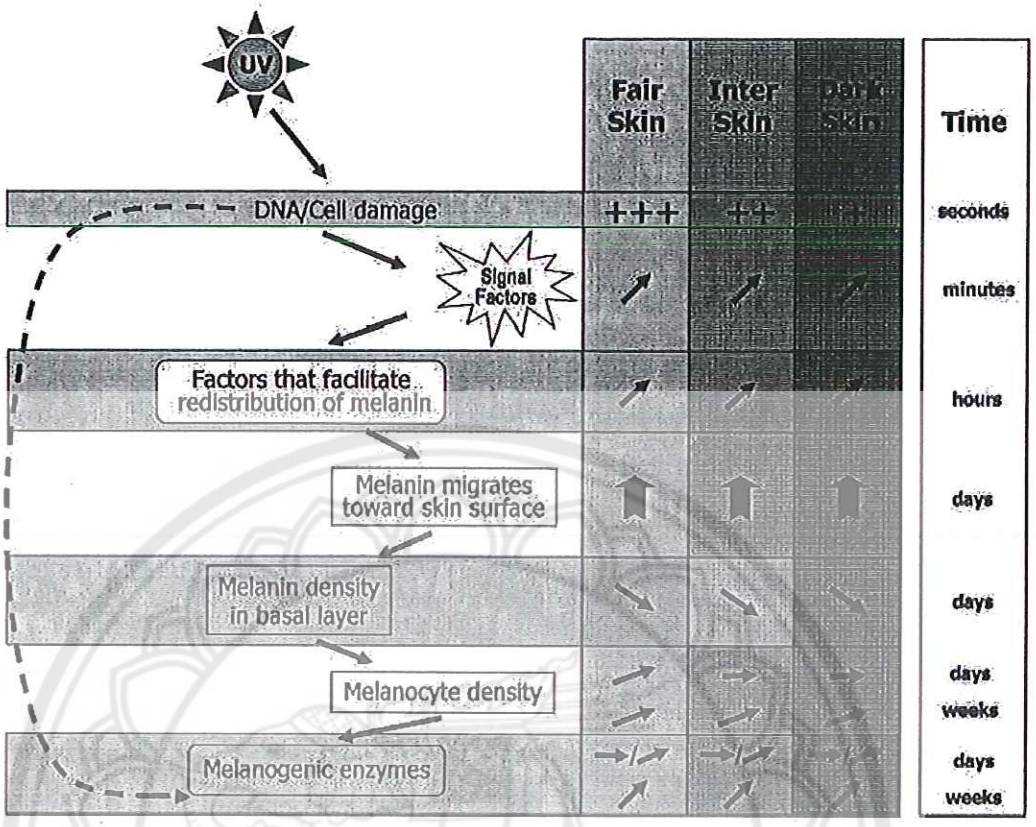

Figure 14 Scheme summarizing the effects of UV on skin in a time-dependent manner. Intensities of effects on Fair, Intermediate and Dark skin are shown by the direction of the arrows

Source: Beer, 2007

Skin aging

Skin aging can be divided into two basic processes, intrinsic aging (or chronological aging) and photoaging (Gilchrest, 1989). Photoaging describes premature skin aging in chronically photodamaged skin. If habitually sun-exposed skin in the elderly is compared with skin from the sun by clothing, the exposed skin appears more aged. Intrinsic aging is characterized by smooth, dry, pale and finely wrinkled skin. On the other hand, photoaging is characterized by severe wrinkling and pigmentary changes, such as solar lentigo and mottled pigmentation on exposed areas such as the face, neck and forearm.

Similarities between intrinsic aging and photoaging

Skin, like all organs, ages with the passage of time. Compared with photodamaged skin, sun-protected aged skin appears thinner, more evenly pigmented, laxer, and more finely lined. Often, photoaging and intrinsic aging have been considered separate entities. However, recent evidence indicates that they share some important molecular features. Photoaging is the superposition of UV irradiation from the sun on intrinsic aging. To exert its biological effects, UV irradiation must be absorbed by molecules (chromophores) in the skin, and the absorbed energy must be converted into chemical reactions. Depending on the chromophore, absorbed energy may cause direct chemical modification of the chromophore itself, or the energy may be transferred from the chromophore to another molecule, which undergoes chemical modification. For example, UV-B absorption by DNA causes cross-linking of adjacent pyrimidines, whereas UV-A absorbing skin chromophores transfer energy to oxygen to generate ROS (reactive oxygen species), which oxidize cellular constituents including proteins, lipids, and DNA. Photoaging is mediated by direct UV absorption and ROS-mediated photochemical reactions. The cause of intrinsic aging is far less clear than that of photoaging. Many theories have been advanced to explain intrinsic aging. One, the free radical theory, states that aging results from accumulation of cellular damage that results from excess ROS that are generated as a consequence of oxidative metabolism (Sohal, 1996; Hensley, 2002). Age associated cellular damage includes oxidation of DNA resulting in mutations, oxidation of proteins resulting in reduced function, and oxidation of membrane lipids resulting in reduced transport efficiency and possibly altered transmembrane signaling. The main source of excess ROS implicated in aging is mitochondrial oxidative energy generation. As a result of accumulated damage, the aged cell has reduced antioxidant capacity (Chamougrand, 2001; Ma, 2002), further exacerbating ROS-mediated damage and the aged phenotype (Herman, 2001). Given the central role of ROS in both photoaging and intrinsic aging, it is possible that the 2 processes have common molecular mediators. Transcription factor AP-1 is a critical mediator of acute photodamage that is involved in both over expression of MMPs and reduction of type I procollagen. In human skin, AP-1 activity is limited by c-Jun expression, since c-Fos is continuously expressed (Fisher, 1998). Interestingly, while c-Fos expression in young (18-28 years old) and aged (>80 years

old) skin does not differ; c-Jun expression is elevated in aged compared with young skin. Both c-Jun mRNA and protein levels are elevated in aged skin, a finding similar to that in UV-irradiated skin. In addition, the activity of the upstream activator of c-Jun, c-Jun N-terminal kinase, is also elevated in aged compared with young skin (Chung, 2000). These data suggest that AP-1 activity is increased in aged skin. In support of this possibility, AP-1–regulated MMP-1 and MMP-9 activities are also increased in aged human skin in vivo (Varani, 2000). Increased MMP activity would be expected, over time, to degrade dermal connective tissue. Consistent with this view, insoluble partially degraded collagen, as a percentage of total collagen, is increased 4-fold in aged compared with young human skin (data not shown; unpublished data, 2002). This increased percentage of partially degraded collagen in aged skin is similar to that observed in photoaged skin compared with sun-protected skin. These data indicate that imperfect repair of elevated MMP mediated collagen breakdown in aged skin, as in UV-irradiated skin, results in accumulation of collagen fragments. Transmission electron microscopy reveals the fragmented nature of collagen fibrils in photoaged and aged human skin in vivo.

UV irradiation activates cell surface growth factor and cytokine receptors

Signal transduction pathways that are activated by UV irradiation in human skin are showed in Figure 15. Ultraviolet irradiation causes activation of cell surface cytokines and growth factor receptors. Epidermal growth factor (EGF), interleukin (IL) 1, and tumor necrosis factor α (TNF-α) receptors are activated within 15 minutes following UV exposure (twice the minimal erythema dose) in human skin in vivo. Functional activation of these receptors requires stimulation of distinct tyrosine kinase activities (Ullrich, 1990). This addition of phosphate groups to tyrosine residues on the receptors and their associated adaptor proteins is the initial biochemical event in receptor activation and provides specific docking sites for molecules involved in signal propagation within the cell. The primary mechanism by which UV irradiation initiates molecular responses in human skin is by photochronical generation of ROS. These ROS include superoxide anion, peroxide, and singlet oxygen. The mechanism of receptor activation by UV irradiation is not well understood. One possibility, which is supported by indirect experimental evidence, is that photochemical generation of

ROS oxidize, and thereby inhibit, specific protein-tyrosine phosphatases, which function in opposition to receptor activated protein-tyrosine kinases by removing phosphate group from receptors or their associated adaptor proteins. The result would be a net increase in receptor phosphorylation (activation). This mechanism of UV activation of cell surface receptors is supported by several publications although direct evidence that protein-tyrosine phosphatases regulate the activation state of cell surface receptors is lacking.

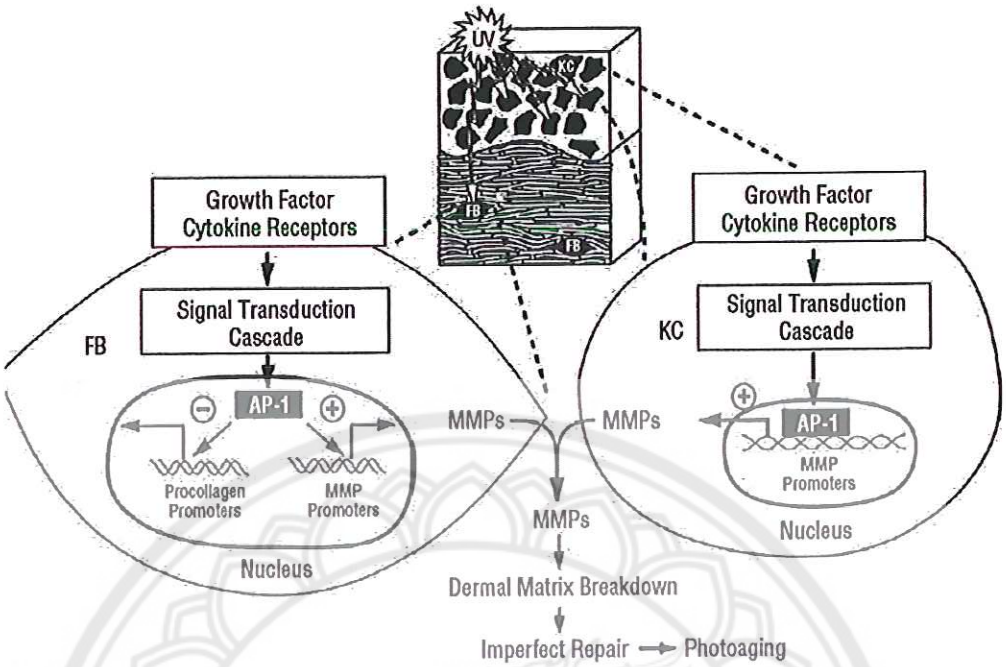

Figure 15 Model describing solar UV irradiation damage to skin connective tissue. Ultraviolet irradiation (jagged arrows) activates growth factor and cytokine receptors on the surface of keratinocytes (KC) and fibroblasts (FB). Activated receptors stimulate signal transduction cascades that induce transcription factor AP-1, which stimulates transcription of matrix metalloproteinase (MMP) genes. In fibroblasts, AP-1 also inhibits procollagen gene expression. Matrix metalloproteinases are secreted from keratinocytes and fibroblasts and break down collagen and other proteins that comprise the dermal extracellular matrix. Imperfect repair of the dermal damage impairs the functional and structural integrity of the extracellular matrix. Repeated sun exposure causes accumulation of dermal damage that eventually results in characteristic wrinkling of photodamaged skin

Source: Gary, 2002

UV irradiation activates NADPH oxidase, which generates hydrogen peroxide

The mechanism of UV irradiation activates cell surface receptors, as does ligand binding, and triggers downstream signal transduction pathways. Several studies have demonstrated that UV irradiation stimulates production of hydrogen peroxide, which induces multiple signaling pathways, although the mechanism(s) by which hydrogen peroxide acts is not clear. In human skin in vivo and human keratinocytes, hydrogen peroxide levels are increased within 15 minutes following UV irradiation and continue to accumulate for approximately 60 minutes after UV exposure. It is important to appreciate that this generation of hydrogen peroxide after UV exposure is distinct from photochemical generation of ROS described above, which occurs only during UV exposure and reduce following UV exposure. Phagocytes contain a multi-subunit complex, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which catalyzes the reduction of molecular oxygen to superoxide anion. The superoxide produced is quantitatively converted to hydrogen peroxide, which is less damaging to cells and serves as a cosubstrate for peroxidases. In turn, hydrogen peroxide can be converted to other ROS, including hydroxyl radical and singlet oxygen. Human skin and human keratinocytes also express NADPH oxidase subunits, and contain NADPH oxidase activity that is induced following UV exposure. In keratinocytes, NADPH oxidase activity is induced 2-fold within 20 minutes following UV exposure (Babior, 2002).

UV-induced MMPs degrade skin collagen

Ultraviolet-induced MMP-1 initiates cleavage of fibrillar collagen (type I and III in skin) at a single site within its central triple helix. Once cleaved by MMP-1, collagen can be further degraded by elevated levels of MMP-3 and MMP-9 (Sternlicht and Werb, 2001). Metalloproteinase 1, MMP-3, and MMP-9 activities have been shown to colocalize with collagen in the dermis, following UV irradiation of human skin in vivo. Type I collagen molecules are stabilized by intermolecular covalent cross-links. Depending on the extent of degradation, partially degraded collagen can remain cross-linked within the insoluble collagen matrix. These insoluble collagen fragments are susceptible to proteolytic cleavage, in vitro, by proteases with broad specificity such as chymotrypsin. When sun-protected human skin was irradiated and

biopsy performed, results showed the level of partially degraded collagen 24 hours following UV irradiation increased 3-fold. Thus, UV-induced MMPs degrade skin collagen and thereby impair the structural integrity of the dermis. In the absence of perfect repair, MMP-mediated collagen damage is expected to accumulate with each successive UV exposure. Such cumulative collagen damage is likely a major contributor to the phenotype of photoaged human skin.

UV irradiation inhibits production of type I and type III procollagen

In addition to degrading mature dermal collagen, UV irradiation impairs ongoing collagen synthesis, primarily through down-regulation of type I and type III procollagen gene expression (Fisher, 2000). Two mechanisms contribute to reduced procollagen gene expression. UV irradiation induces the transcription factor AP-1. By binding and sequestering factors that are part of a transcriptional complex required for procollagen transcription, AP-1 interferes with collagen production. Transcription factor AP-1 has also been shown to decrease collagen synthesis by blocking the effects of transforming growth factor β (TGF-β), a major profibrotic cytokine, and sequestering one of the signaling proteins it activates both directly and indirectly. Ultraviolet irradiation also interferes with TGF-β dependent type I procollagen gene expression by downregulating type II TGF-β receptor, within 8 hours of irradiation, rendering the cells unresponsive to TGF-β effects. In cultured human fibroblasts, UV-induced down regulation of type II TGF-β receptor and subsequent loss of TGF-β responsiveness results in substantial reduction of type I procollagen gene expression. These data suggest that down-regulation of type II TGF-β receptors, in addition to AP-1–mediated transcriptional repression, contribute to reduced procollagen gene expression observed in human skin in vivo, following UV irradiation.

Skin pigment protects against UV-induced response that lead to collagen degradation

It is well established that skin pigment provides a significant degree of protection against actinic damage. For a given exposure to UV irradiation, persons with less pigment (lighter skin color) will exhibit greater erythema and less tanning than persons with more pigment (darker skin pigment). This inverse relationship between skin color and UV-induced skin reddening (sunburn) forms the basis of the skin phototype classification system. This classification system identifies 6 skin

phototypes, with light-skinned persons who sunburn easily and do not tan (classified as skin phototype I) and dark-skinned persons who do not sunburn and tan readily as phototype VI. The clinical observation support that photoaging is less severe in the darkly pigmented population compared with the lightly pigmented population (Otonne, 2002).

Damaged collagen as a regulator of type I procollagen synthesis implications for photoaging and chronological aging

In addition to reduction of type I procollagen synthesis following immediate UV irradiation as described earlier, ongoing procollagen synthesis is significantly reduced in severely photodamaged and chronologically aged skin, independent of recent UV exposure (Griffiths, et al., 1993). Mechanisms underlying the sustained reduction in procollagen synthesis are not fully understood. In full-thickness photoaged skin, total collagen content is only modestly reduced (Bernstein and Uitto, 1996). However, procollagen gene and protein expression are substantially reduced in approximately the upper one third of the dermis (Talwar, et al., 1995). This restricted localization of procollagen reduction likely reflects the depth of penetration of UV irradiation into the skin, which mitigates more extensive reduction of total collagen. In photodamaged skin, the number of fibroblasts in vitro is similar to that in sun-protected skin of the same subjects, and the capacity to synthesize type I procollagen is the same for fibroblasts cultured from both areas (Varani, et al., 2000). Thus, the reduced procollagen observed in photodamaged skin likely results from down-regulation of fibroblast procollagen synthesis by factors within the dermal milieu rather than from inherent alterations in the fibroblasts. Interestingly, exposure of cultured fibroblasts from either photodamaged or sun-protected skin to partially degraded type I collagen, produced by in vitro treatment of collagen with a mixture of MMPs from human skin, inhibited procollagen synthesis. These results suggest that elevated levels of degraded collagen observed in photodamaged skin act to down-regulate type I procollagen synthesis. Of the different MMPs, MMP-1 was the most effective collagenase, followed by MMP-8 and MMP-13 (Varani, et al., 2001). Gelatinolytic enzymes (MMP-2 and MMP-9) did not degrade intact collagen and did not inhibit procollagen synthesis, but the combination of MMP-1 and MMP-9 broke down collagen to small peptides. Interestingly, these small fragments did not inhibit

procollagen synthesis, but rather the larger breakdown fragments of type I collagen negatively regulated its synthesis. Taken together, these data suggest that the high molecular weight fragments of type I collagen serve as negative regulators of type I collagen synthesis and that further breakdown of MMP-1–cleaved collagen by MMP-9 can alleviate this inhibition. Thus, UV-induced MMPs damage the dermis by 2 related mechanisms: direct degradation of collagen and indirect inhibition of collagen synthesis by MMP-generated collagen degradation products. In contrast to photodamaged skin, in aged sunprotected skin, both the number of fibroblasts and their capacity to synthesize type I procollagen are reduced compared with young skin. In addition, aged skin, similar to photoaged skin, contains elevated levels of partially degraded collagen. Therefore, it is likely that the inhibitory effects of collagen fragments observed in photoaged skin are also operative in aged skin and are superimposed on an intrinsic decline in collagen synthetic activity. Reduced mechanical tension may also contribute to diminished number of fibroblasts in aged skin because the loss of mechanical tension results in increased apoptosis in model cell systems (Huang and Ungber, 2000). How MMP-damaged collagen functions to inhibit new collagen synthesis in vivo is not known, but collagen synthetic activity is regulated by mechanical tension on fibroblasts resulting from attachment to a firm substratum. Fibroblasts exert contractile forces on the collagen extracellular matrix, and the physical resistance of the matrix to this contraction generates mechanical tension on the fibroblast. The rate of collagen synthesis is proportional to the level of mechanical tension. Damaged collagen fibrils are more pliable than native fibrils. As fibroblasts interact with damaged collagen fibrils, the cells experience less resistance and therefore less mechanical tension, resulting in reduced procollagen synthesis. How mechanical tension regulates procollagen synthesis is poorly understood, although many cellular functions are affected by mechanical tension, including cell surface receptor activation, signal transduction, gene expression, and cell growth (Lambert, et al., 2001).

Adaptive Cellular Responses in Photoaging

Adaptation is defined as an anatomical structure, a physiological process, or a behavior pattern that makes an organism more fit to survive and reproduce in competition with other members of its species. Mechanisms of adaptation of skin to photo-oxidative stress include pigmentation/skin color, epidermal hyperplasia, and enhanced anti-oxidative defense. The cell can respond to photo-oxidative stress with changes in the non-enzymatic and enzymatic antioxidant defense system, with termination of replication and senescence, DNA repair or apoptosis. Cellular antioxidative protective mechanisms comprise the non-enzymatic low molecular weight antioxidants ascorbic acid (Vitamin C) and tocopherol (Vitamin E), which cannot be synthesized by humans, and different antioxidants that are synthesized by the human body like glutathione, ubiquinone and others. The antioxidative enzymes comprise mainly the superoxide dismutases (SOD), glutathione peroxidases (GSX), catalase (CAT), GSH synthase and GSSG reductase with different subcellular location and substrate specificity that act interrelated in a specific sequence to detoxify the dangerous reactive oxygen species generated by UV-irradiation (Figure 16) and other mechanisms.

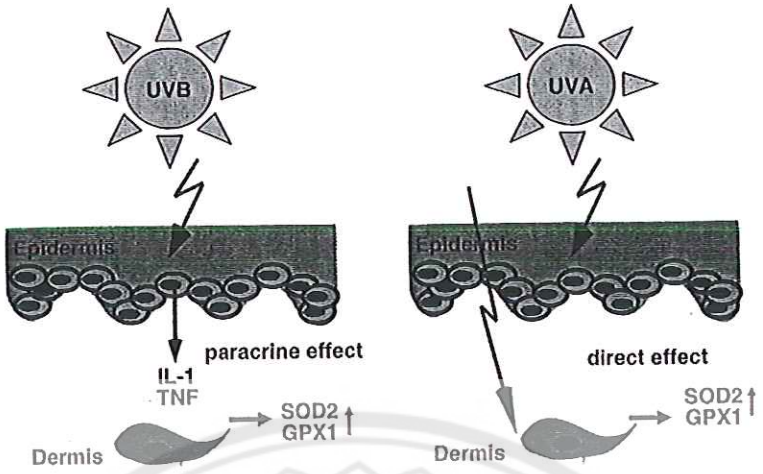

Figure 16 **The adaptive antioxidative response of dermal fibroblasts differs due to the UV spectrum. Repetitive UVA irradiation leads to an adaptive response via the induction of SOD2 and GPX1 expression and activity. The adaptive response of the induction of SOD2 and GPX1in fibroblasts after UVB is mediated by a paracrine activation from the epidermal keratinocytes**

Source: Wlaschek, et al., 2007

1. Detoxifying enzymes

The superoxide anion O^{-}_2, one of the prominent radical species that could give rise to other reactive compounds, is converted to H_2O_2 by the action of the enzyme superoxide dismutase (SOD). H_2O_2 is in turn converted to H_2O and O_2 by the enzyme catalase (CAT). Alternatively, H_2O_2 may be converted to H_2O by the action of glutathione peroxidases (GPX) in many cells. Antioxidative activity was also shown for the reducing enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR), which restore the intracellular antioxidants glutathione and thioredoxin. The enzymes work in a cooperative manner, but not all of them are simultaneously necessary for an effective antioxidative shield. For example, the lack of glutathione peroxidase/reductase in Drosophila is substituted by the activity of the thioredoxin system (Sohal, et al., 1990; Kanzok, et al., 2001). If the oxidative damage caused by excess ROS contributes to aging and lifespan determination as suggested by the oxidative damage theory of aging, enhancing the enzymatic detoxification system may

slow aging and extend lifespan. But in stress situations, when enzymatic activities become insufficient, increased activities of these anti-oxidant enzymes can have beneficial effects.

1.1 Glutathione (GSH)

Glutathione is the most abundant low molecular weight thiol, synthesized in most cell types, where it is in the millimolar range and plays a key role in maintaining the intracellular redox equilibrium. Cellular glutathione exists mainly in the reduced thiol form, GSH, while 1-5% of the total GSH pool is in the oxidized disulfide form, GSSG. However, under oxidizing conditions, depletion of GSH and a consequent decrease in the GSH/GSSG ratio may occur. Besides its antioxidant properties, which are mediated by peroxidase-coupled reactions, GSH may also modulate cell signaling and a variety of cellular events. Through thiol-disulfide exchange reactions, GSH is responsible for protein modifications that regulate cellular function and survival. Under conditions of oxidative stress, the degree of GSH depletion, the GSH pool affected (cytosol, endoplasmic reticulum, mitochondria or nucleus) and the types of stimuli, are fundamental in determining the final outcomes (Dickinson and Forman, 2002; Circu and Aw, 2008; Biswas and Rahman, 2009; Forman, et al., 2009; Yuan and Kaplowitz, 2009). Another important mechanism through which GSH can modulate the activity of several enzymes and signaling transducers, both under oxidant stress, and in physiologically relevant conditions.