Vitamins as Hormones

Abstract

Vitamins A and D are the first group of substances that have been reported to exhibit properties of skin hormones, such as organized metabolism, activation, inactivation, and elimination in specialized cells of the tissue, exertion of biological activity, and release in the circulation. Vitamin A and its two important metabolites, retinoldehyde and retinoic acids, are fat-soluble unsaturated isoprenoids necessary for growth, differentiation and maintenance of epithelial tissues, and also for reproduction. In a reversible process, vitamin A is oxidized in vivo to give retinaldehyde, which is important for vision. The dramatic effects of vitamin A analogues on embryogenesis have been studied by animal experiments; the clinical malformation pattern in humans is known. Retinoic acids are major oxidative metabolites of vitamin A and can substitute for it in vitamin A-deficient animals in growth promotion and epithelial differentiation. Natural vitamin A metabolites are vitamins, because vitamin A is not synthesized in the body and must be derived from carotenoids in the diet. On the other hand, retinoids are also hormones – with intracrine activity – because retinol is transformed in the cells into molecules that bind to and activate specific nuclear receptors, exhibit their function, and are subsequently inactivated. The mechanisms of action of natural vitamin A metabolites on human skin are based on the time- and dose-dependent influence of morphogenesis, epithelial cell proliferation and differentiation, epithelial and mesenchymal synthetic performance, immune modulation, stimulation of angiogenesis and inhibition of carcinogenesis. As drugs, vitamin A and its natural metabolites have been approved for the topical and systemic treatment of mild to moderate and severe, recalcitrant acne, photoaging and biologic skin aging, acute promyelocytic leukaemia and Kaposi’s sarcoma. On the other hand, the critical importance of the skin for the human body’s vitamin D endocrine system is documented by the fact that the skin is both the site of vitamin D₃- and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]-synthesis and a target organ for 1,25(OH)₂D₃. 1,25(OH)₂D₃ is not only essential for mineral homeostasis and bone integrity, but also for numerous further physiologic functions including regulation of growth and differentiation in a broad variety of normal and malignant tissues, including cells derived from prostate, breast and bone. In keratinocytes and other cell types, 1,25(OH)₂D₃ regulates growth and differentiation. Consequently, vitamin D analogues have been introduced for the treatment of the hyperproliferative skin disease psoriasis. Other newly detected functions of vitamin D analogues include profound effects on the immune system as well as protection against cancer and other diseases, including autoimmune and infectious diseases, in various tissues. Current investigation of the biological effects of vitamin D analogues are likely to lead to new therapeutic applications that, besides cancer prevention, may include the prevention and treatment of infectious as well as of inflammatory skin diseases. This review summarizes existing knowledge on vitamins A and D, the major vitamin-hormones of the skin.

Vitamin A, natural retinoids and the skin

The term “Retinoids” includes both naturally occurring molecules and synthetic compounds showing biological activities, which are characteristic for vitamin A. The current definition of retinoids as it was defined by the IUPAC-IUB Joint Commission on Biochemical Nomenclature in 1982, i.e., the “four isoprenoid units joined in a head-to-tail manner” structure, does not require a chemical analogy to vitamin A. In general, retin-
oids bind and activate specific nuclear receptors, although this may not be a necessary precondition for parts of their action. The first part of this review is dedicated to existing knowledge on the activity of natural retinoids, namely vitamin A and its metabolites, as hormones.

Vitamin A (retinol) and its two important metabolites, retinaldehyde and retinoic acids, are fat-soluble unsaturated isoprenoids necessary for growth, differentiation and maintenance of epithelial tissues, and also for reproduction. In a reversible process, retinol is oxidized in vivo to give retinaldehyde, which is important for vision. The normal plasma concentration of vitamin A in humans is 0.35–0.75 µg/ml [1].

Retinoic acids are a major oxidative metabolite of retinol and can substitute for retinol in vitamin A-deficient animals in growth promotion and epithelial differentiation. However, they cannot substitute for retinol completely in maintaining reproduction. The stereoisomers all-trans-retinoic acid (atRA), 13-cis-retinoic acid (13cRA) and 9-cis-retinoic acid (9cRA) are normal constituents of human serum [2]. Unlike the retinol esters, which represent the storage form for retinol and are deposited in the liver, retinoic acids are not stored but are rapidly excreted. The normal levels in human plasma are 0.55–1.20 ng/ml for atRA, 0.80–2.40 ng/ml for 13cRA and traces for 9cRA [3].

Endogenous retinoids are unlikely to be involved in the pathogenesis of common skin diseases, such as acne and psoriasis [1,3]; in contrast, hypervitaminosis A is associated with a broad spectrum of symptoms resembling the mucocutaneous side effects of oral treatment with synthetic retinoids. Humans require 0.8–1 mg or 2400–3000 I.U. vitamin A per day (1 I.U. = 0.3 µg). However, vitamin A intoxication only occurs when daily dietary intake of vitamin A exceeds 18000 to 60000 I.U. per day in children and 50000 to 100000 I.U. in adults, given over a period of several months [4]. In the case of restricted liver metabolic capacity, symptoms of intoxication may appear much earlier, within a few months, and when smaller doses are taken (10000 I.U. per day). Hypervitaminosis A is signaled by an increase in retinol ester levels (normal value is 5–8 % of retinol) in serum. The retinol values rarely increase. Pregnant women and women of childbearing age should not exceed oral intake of 8000 to 10000 I.U. vitamin A per day.

Molecular mechanisms of action ▼

Although retinol is assumed to enter the cells by non-receptor-mediated endocytosis [5], the exact mechanism of retinoid-induced membrane-associated signal transduction is still unknown. Intracellularly, atRA and 9cRA are transported by cytosolic proteins, bind to specific nuclear receptors, induce expression of genes which bear specific DNA sequences recognizing the retinoid/receptor complex [6,7], and are finally inactivated [8]. These pathways have been well investigated for atRA, but they may not be valid for all retinoid compounds. The discovery of the specific cellular retinoid binding proteins and the nuclear retinoid receptors has extended the understanding for the broad spectrum of the biological activity of retinoids [9]. These observations have also thrown light on the complex interactions between retinoids and further hormonal signal transduction molecules. atRA is transported by the cellular retinoic acid-binding proteins (CRABP) from the cytoplasm to the nucleus. The dominant CRABP in the skin is CRABP II, whereas expression of low quantities of CRABP I was also found. The expression of CRABP II is high-adjusted by the local use of atRA. CRABP II is considered as an early marker of retinoid activity on the skin and probably can control the bioavailability of retinoids.

Retinoid receptors are members of the nuclear receptor superfamily and are classified in two families; the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) [10]. They act as ligand-dependent transcription factors. RARs can bind both atRA and 9cRA with high affinity, while RXRs selectively interact with 9cRA. Both types of retinoid receptors exhibit α, β and γ subtypes, whereby each type includes a number of isoforms. The retinoid nuclear receptor complex activates genes, which possess specific short DNA sequences in their promoter regions, known as retinoid-response elements. The fact that not all retinoids combine biological activity and affinity for and/or activation of the receptors has led to suggestions for existence of additional unknown retinoid signaling pathways. 13cRA shows low affinity for RARs and 14-hydroxy-retinol, which specifically induces lymphocyte proliferation, and does not bind to or activate retinoid receptors [10]. However, the knowledge that retinol activation requires its metabolism to the receptor-binding retinoic acids, particularly atRA, and the elucidation of the mechanism of 13cRA action on human sebocytes corroborate the significance of retinoid receptors for biological retinoid activity; 13cRA is a pro-drug, activated in human sebocytes through a selective intracellular isomerization to high levels of atRA and subsequent binding to RARs [11].

Retinoid receptors bind retinoids in the form of dimers, as homodimers (RXR/RXR) or heterodimers (RAR/RXR) [10]. Heterodimers can be also formed between RXR and the vitamin D receptor (VDR/RXR) as well as between RXR and 13 other members of the nuclear receptor superfamily. This discovery has thrown light on the complex interactions between retinoids and further hormonal signal transduction molecules. Following this concept, RXR selective retinoids may influence vitamin D and other nuclear hormone target genes, in addition to the RXR responsive ones. On the other hand, vitamin D and other nuclear hormones may also regulate RXR responsive genes. Clinically, combinations of RXR selective retinoids and vitamin D derivatives may potentiate the expected therapeutic result and decrease toxicity of each single compound [12].

RARs and γ and RXRs, α, β, γ are expressed in epidermal keratinocytes of the stratum granulosum, follicular keratinocytes, sebocytes, and endothelial cells, while only the RXR γ isotype is present in melanocytes, fibroblasts and inflammatory cells [11,13–16] (Fig. 1).

Natural retinoids are not only vitamins but also hormones ▼

Natural retinoids are vitamins because retinol is not synthesized in the body and must be derived from carotenoids in the diet [17]. On the other hand, retinoids are also hormones – with intracrine activity [18] – because retinol is transformed in the cells into molecules that bind to nuclear receptors, exhibit their activity, and are subsequently inactivated (Fig. 1). The intracrine concept implies an intracellular control of the amount of ligand available to nuclear receptors. Because the intracellular amount of the ligand required for exhibiting normal activity is low, homeostasis is guaranteed by the ability of the system to inactivate excessive retinoid amounts. Molecules shown to control the bioavailability of retinoic acid are cytochrome P450...
isoenzymes and CRABP II [19]. Recently, 13cRA was shown to competitively inhibit 3α-hydroxysteroid oxidation by retinol dehydrogenase, a mechanism that may contribute to an antiandrogenic effect of 13cRA in sebaceous glands [20].

Pharmacokinetics and cutaneous metabolism

Transport mechanisms and pharmacokinetics of retinol are known to a large extent [17, 21]. Retinol is transported in plasma by the specific retinol-binding protein (RBP) to the peripheral tissues. The intracellular absorption of retinol takes place via diffusion without the assistance of receptors. Intracellularly, cellular retinol-binding proteins (CRBP) take over the function of the RBP. Retinol is naturally oxidized to retinaldehyde by CRBP I and the NADP⁺-dependent retinol dehydrogenase. β-Carotenes, which are taken up by food, can be converted to retinaldehyde. Retinaldehyde is further oxidized with the help of the NAD⁺-dependent retinol dehydrogenase to atRA, which is the most active natural retinoid. Excessive retinol is converted intracellularly with the help of the enzyme lecithin retinol acetyl transferase to retinyl esters, which can be oxidized with the help of the retinyl ester hydrolase back to retinol.

Retinoid metabolism in human skin is likely to be a cell-specific event, since sebocytes exhibit a distinct metabolic pattern compared to epidermal keratinocytes [15]. Excess retinol is mainly esterified [22]; application of retinol to human skin in vivo does not lead to measurable retinoic acid levels [23]. Human keratinocytes in vivo regulate the levels of intracellular atRA by induction of retinoic acid 4-hydroxylase [24], preventing the accumulation of atRA in the epidermis. Human keratinocytes in vitro rapidly take up and also initially convert retinol to retinyl esters and then to low amounts of atRA with time [8, 25]. In contrast to atRA, retinol, retinaldehyde, 9cRA and 13cRA are not able to regulate their own hydroxylation. 3,4-Didehydroretinol can also be detected [26]. However, ester formation, especially of retinyl oleate (18:1) and retinyl palmitate (16:0), remains the main route by which excess retinol is also handled by human keratinocytes in vitro.

atRA is photochemically unstable. With its topical application it is partially isomerized into 13cRA and 9cRA as well as to a number of further retinoid metabolites in the epidermis [26]. Approximately 80% of the substance remains at the skin surface. Topically applied retinoids penetrate the epidermis through the stratum corneum and the follicular epithelium, whereby their penetration index is dependent on the basis of the preparation used. The diffusion of retinoids in the stratum corneum is rapid; they form within few minutes a substance reserve in the horny layer. Penetration into the deeper epidermis and into the dermis is usually slower. The percutaneous absorption of atRA is between 0.1 and 7.2%. Inactivation of topical atRA seems to take place via the induction of cytochrome P450-retinoic acid hydroxylase activity, which catalyzes the metabolism to 4-hydroxyretinoic acid. Another inactivation product is 5,6-epoxyretinoic acid. The metabolic pathways of 13cRA and 9cRA in vivo are still unclear, whereas – in addition to its possible isomerization to atRA [11] – the 4-oxo metabolism of 13cRA could be another activation pathway, since 4-oxoretinoids were shown to be functionally active in human keratinocytes and fibroblasts by their ability to induce changes in gene expression [27]. Alternatively, 13cRA has been suggested to act in a receptor-independent manner by influencing cellular signaling pathways by direct protein interactions as demonstrated with other retinoids or by enzyme inhibition [20]. Topically applied 13cRA is partially converted by UV light to atRA [21].

Cellular mechanisms of action

The cellular mechanisms of action of natural retinoids are based on the time- and dose-dependent influence of morphogenesis, epithelial cell proliferation and differentiation, epithelial and mesenchymal synthetic performance, immune modulation, stimulation of angiogenesis and inhibition of carcinogenesis. The dramatic effects of retinoids on embryogenesis were studied by animal experiments; the clinical malformation pattern in humans is known [28, 29].

Retinoids promote cell proliferation in normal epidermis by shortening the mitotic phase of cell cycle, but act towards normalization in hyperproliferative epithelia. Proliferation of rapidly proliferating keratinocytes, such as psoriatic ones, is down-regulated by retinoids. In vitro, atRA was shown to either stimulate or inhibit epidermal keratinocyte proliferation, depending on the growth-culture conditions. Stimulation of keratinocyte proliferation is associated with induction of cAMP, epidermal growth factor (EGF)-receptor binding, protein kinase C and tumor growth factor (TGF)-α [30, 31]. Epidermal thickening with voluminous stratum spinosum and stratum granulo-
sum is the histological correlate. On the other hand, TGF-β2-regulated inhibition of EGF-binding to its receptor leads to downregulation of cell growth. The effect of atRA on EGF receptor-binding is on a region of the EGF promoter regulated by RAR. A biphasic effect of natural retinoids on human sebocytes is also detected. Under normal conditions, atRA and 13cRA inhibit sebocyte proliferation [32], while atRA substitutes the nutritional effect of retinol after cell maintenance under retinol-depleted conditions [33]. Parallel to these effects, retinoids alter terminal keratinocyte differentiation towards a metaplastic, non-keratinizing, mucosa-like epithelium, whereas the glycosylation pattern of normal skin treated with atRA resembles that of a mucosal epithelium, with reduction of tonofilaments, decreased cohesiveness of the stratum corneum, impaired function of the permeability barrier, and increased transepidermal water loss, causing the keratolytic effect of retinoids in hyperkeratotic disorders. In contrast, oral and topical retinoids stimulate terminal differentiation of human epidermal cells, e.g., in the psoriatic plaque [17, 21]. In vitro, most markers of terminal differentiation (loricrin, transglutaminase, involucrin, filaggrin, keratins 1 and 10) are down-regulated by atRA in a dose-dependent manner and keratins 19 and 13, markers of nonstratified and wet stratified epithelia, respectively, are induced [34]. In contrast, natural atRA restores the architecture of the “epidermis” in the air-medium interface model, which exhibits excessive hyperkeratosis in vitamin A-depleted medium [35].

atRA up-regulated hyaluronan synthase 2 (Has2) gene expression 4-fold within two hours in rat epidermal keratinocytes and even 8-fold in HaCaT human immortal keratinocytes [36]. Hyaluronan is an abundant and rapidly turned over matrix molecule between the vital cell layers of the epidermis and subject to large concentration changes associated with keratinocyte proliferation, migration and differentiation induced by paracrine and endocrine factors like EGF and atRA. The first 10 kbp of the human Has2 promoter were scanned in silico and in vitro for potential retinoic acid response elements (RAREs) and a complex of two of these RAREs was found approximately 1200 bp upstream of the transcription start site. Moreover, chromatin immunoprecipitation assays using antibodies against nine nuclear proteins monitored atRA-dependent binding of RAR, RXR, mediator protein and RNA polymerase II and also histone 4 acetylation to a promoter region containing the complex RARE. This indicates that the human Has2 gene is a potent primary EGF and atRA responding gene with a complex regulation.

Retinoic acids exhibit earlier and stronger biological effects on keratinocytes than retinol, probably due to their early high cellular accumulation and their slower inactivation [8, 17]. These findings support the assumption that the intensity of retinoid signaling is dependent, in part, on the intracellular concentrations of retinoic acid. This assumption is supported by the tight autoregulatory mechanism in human keratinocytes offering protection against excessive accumulation of cellular retinoic acid, e.g., the induction of CRABP II [14, 17]. Most actions of atRA are recognized to be mediated through activation of RARs, whereas in epithelial skin cells RARs modulate cell proliferation, while RXRs rather influence cell differentiation [11]. Retinoids regulate proliferation and differentiation of skin epithelial cells towards a homeostatic status [17, 21].

13cRA is the most effective retinoid in reducing sebaceous gland size (up to 90%), by decreasing proliferation of basal sebocytes, and suppressing sebum production in vivo. Marked decrease of wax esters, light decrease of squalene and relative increase of cholesterol concentration has been detected in skin surface lipids. Orally administered 13cRA was also shown to decrease glyceride fraction, whereas free sterols and total ceramides were increased in comedonal lipids [37]. 9cRA was found to be inferior to 13cRA in sebum suppression [38]. In vitro studies confirmed the pronounced, direct inhibitory effects of 13cRA on proliferation, lipid synthesis, and differentiation of human sebocytes in vitro [39–41]. Current data indicate that in sebocytes, 13cRA causes inhibition of cell proliferation after intracellular metabolism to atRA by a RAR-mediated pathway and cell cycle arrest and apoptosis by a RAR-independent mechanism, which contributes to its sebosuppressive effect [11, 42] (Table 1).

Multiple effects of retinoids on the cellular and humoral immunity – probably by activating phospholipase C and phosphokinase C as well as by stimulating the antigen-presenting capacity of the Langerhans cells and inducing ICAM-1 expression on keratinocytes – lead to immune-modulatory effects. 14-Hydroxynoletro-retinol was identified to be an essential growth factor for lymphoblastoid cells [43]. Retinoids can enhance antibody production, increasing peripheral blood T helper cells, but not natural killer cells. Topically applied atRA was shown to prevent Langerhans cell depletion in human epidermis due to UV light [44]. In vitro, cell-surface antigens of T- and natural killer cells have been reported to increase after retinoid exposure [45]. Interaction of retinoids and cytokines has been suggested on account of the stronger differentiation response of HL-60 cells to combined atRA and cytokines, particularly IFN-γ, as compared to the single compounds [46]. At the molecular level, the modulation of RAR expression in chicken T lymphocytes by retinol and atRA indicates that antigen-specific proliferative responses of T lymphocytes may be directly influenced by atRA via modulation of RAR expression [47].

Retinoids also exhibit anti-inflammatory activities. Topical 13cRA was found to be more potent in inhibiting leukotriene B4-induced migration of neutrophils into human skin than atRA [48]. 13cRA and atRA inhibited nitric oxide and tumor necrosis factor-α production by human keratinocytes and reduced inducible nitric oxide synthase mRNA levels [49]. There is some early information concerning the activity of retinoids on the endothelium. Retinoids are probably able to induce

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**Table 1** Effectiveness of 13-cis-retinoic acid on human epithelial cells

<table>
<thead>
<tr>
<th>Cellular function</th>
<th>Effect</th>
<th>Cellular mechanism</th>
<th>Molecular mechanism</th>
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<tbody>
<tr>
<td>Proliferation*</td>
<td>Inhibition</td>
<td>Intracellular isomerization in tretinoin</td>
<td>RAR-mediated</td>
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<tr>
<td>Proliferation/ Apoptosis*</td>
<td>Inhibition/ Induction</td>
<td>Cell cycle arrest</td>
<td>Retinoid receptor- independent</td>
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<tr>
<td>Lipid synthesis*</td>
<td>Reduction</td>
<td>Inhibition of terminal differentiation</td>
<td>RAR- and RXR-mediated</td>
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<tr>
<td>Lipid synthesis</td>
<td>Decreased androgen synthesis</td>
<td>Inhibition of 3α-hydroxysteroid activity of retinol dehydrogenase</td>
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<tr>
<td>Inflammation</td>
<td>Inhibition</td>
<td>Inhibition of the migration of neutrophils</td>
<td>Reduction of metalloproteinase expression</td>
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\*Specific effect on sebocytes. RAR = retinoic acid receptor; RXR = retinoid X receptor
angiogenesis and increase the skin blood-flow. On the other hand, 13cRA was shown to inhibit the proliferation of microvascular endothelial cells growing in vitro, without influencing the expression of HLA-DR and ICAM-1 [50]. Retinoids exhibit a direct pro-differentiating effect on tumor cells [51]. The example of tumor cell differentiation in the translocation-associated acute promyelocytic leukemia of mature neutrophils under the influence of atRA makes possible that retinoids may modulate gene expression in tumor cells. Another possible mechanism of the antitumor effect of retinoids can be discussed in the inhibition of ornithin decarboxylase and of the expression of cytochrome P4501A1.

Toxicology of natural retinoids

Characteristic adverse effects of hypervitaminosis A, such as alopecia, elevation of serum triglycerides, hyperostosis, and extraskeletal calcification, can also be observed under treatment with other oral retinoids [17,21]. During embryogenesis, retinoids are highly teratogenic. Because of these adverse effects, oral retinoid treatment appears today strictly contraindicated in pregnancy, during lactation, and in severe hepatic and renal dysfunction, whereas new restrictive guidelines for the use of 13cRA in acnec treatment have been released in Europe and the U.S.A. in order to avoid pregnancies under the drug [52]. Before drug administration, it is strictly recommended to explain the risk of fetal malformations. Hyperlipidemia, diabetes mellitus, and severe osteoporosis are relative contraindications. Limiting conditions for retinoids are gastrointestinal diseases and patient non-compliance. Co-medication with tetracyclines (cranial hypertension) and high doses of aspirin (potentiation of mucosal damage) should be avoided. The mucocutaneous adverse effects of oral retinoids include skin and mucosal dryness (xerosis, cheilitis, conjunctivitis, urethritis), skin fragility and/or stickiness, retinoid dermatitis, palmoplantar desquamation, pruritus, and hair loss. Apart from embryotoxicity, the incidence and severity of these signs are dose-dependent; they are fully reversible upon discontinuation of treatment.

In contrast to the teratogenic effect of systemic retinoids, topical retinoids have shown no mutagenicity, carcinogenesis and teratogenicity in animals. Also in pregnant animals no influence of topical retinoids on the fetus could be observed. An epidemiological study assessed no increased embryopathy risk under topical retinoid application for the treatment of skin diseases. However, an individual embryopathy risk under topical retinoid application cannot be securely excluded, therefore, the topical application of retinoids should be strictly avoided during the first trimester of pregnancy. Skin irritation, xerosis, scaling and itching of the skin are characteristic cutaneous adverse effects of topical retinoids. They occur, depending on retinoid concentration and the type of the vehicle used, during the first month of treatment and spontaneously vanish under continued application.

Therapeutic indications of natural retinoids

Among natural retinoids, retinoic acids have been approved or are under investigation for the systemic treatment of certain disorders shown in Table 2, whereas almost all natural retinoids are used as topical therapeutic compounds [17,21,54].

Retinoids in wound healing

Retinoid effects on connective tissue metabolism are considered to be of paramount importance for a number of therapeutic indications, including their administration in preventing or repairing skin aging (Fig. 2). In addition, changes in epidermal and vascular functions, as mentioned before, probably contribute to beneficial retinoid effects in the skin. Given the regenerative effects of retinoid use on various components of the skin, it is not surprising that retinoids should be considered for use as wound-protective or wound-healing agents. Retinol and retinoic acids, applied topically, counteract atrophic dermal changes by inducing the synthesis of types I and III procollagen [55,56] and suppressing the major collagen-degrading enzymes in the skin [57]. These retinoid effects on the skin reflect multiple mechanisms. Retinoids directly influence gene transcription and, in addition, alter signaling cascades that regulate gene transcription [58,59]. In the case of metalloproteinase reduction, retinoids not only down-regulate enzyme production at the molecular level in different skin cells [57,60] but also up-regulate tissue inhibitor of metalloproteinases-1 [61], the major metalloproteinase inhibitor in the skin. Retinoids also induce an increased production of water-adsorbing glycosaminoglycans [62].

Along with these specific effects on collagen metabolism, retinoid treatment also stimulates proliferation of dermal fibroblasts, thus fostering additional collagen production. Retinoid-mediated effects on cell function can be seen in human skin after as little as 4–7 days of topical treatment [63] (Fig. 2), although, not surprisingly, marked improvements in connective tissue structure are not observed until much longer [64–66]. atRA used in skin that has been severely damaged by chronic, excessive sun-exposure has been convincingly shown to improve skin appearance [64,65]. More recent studies have shown that intrinsically (chronologically) damaged sun-protected skin is also amenable to improvement through retinoid treatment [66]. Several past studies have demonstrated retinoid efficacy in wound healing [67–70]. In most of the studies where retinoid use has been found beneficial, the biologically active retinoid was applied in a pre-treatment mode. Subsequently, when wounds were formed in the retinoid-treated tissue and in the untreated control tissue, wound-healing was superior in the table.
treated tissue [68, 69]. In studies where retinoids, including retinol and atRA, have been used to treat acutely injured tissue, results have been concurrent. Thus, there is consensus, that use of retinoid improves tissue structure and function in such a way as to increase the wound-healing capacity in tissue that subsequently suffers injury. There is, however, no consensus on the value of retinoid application in the treatment of acutely-injured tissue. However, several experimental studies have shown that healthy skin heals rapidly following acute wounding, and under such conditions, pre-treatment with atRA is of limited value. Benefit from retinoid pretreatment is achieved in at-risk skin – i.e., skin that has pre-existing defects that make it susceptible to chronic ulcer formation [68, 70]. Indeed, in most of the studies that have shown a beneficial effect of retinoid use in a pre-treatment mode, the predominant end-point has been a reduction in the time to wound closure [68–70]. Other studies have shown, in addition, that retinoid pretreatment increases the strength of the healed wound. Where retinoid use increases wound strength, there is always a concomitant increase in collagen production [67].

Vitamin D3 and the skin

The skin represents one of the key tissues of the human body’s vitamin D endocrine system, which is of critical importance for a broad variety of independent physiological functions [71–74]. It is well known that 1,25(OH)2D3, the biologically most active naturally occurring vitamin D3 metabolite, is essential for mineral homeostasis and bone integrity [71]. The critical importance of the skin for the human body’s vitamin D endocrine system is documented by the fact that the skin is, first the site of vitamin D3 synthesis, and second a target organ for vitamin D3 metabolism in human skin

A photochemical reaction with maximum spectral effectiveness at about 297 nm results in the generation of previtamin D3 from 7-dehydrocholesterol (provitamin D3, 7-DHC) in basal and suprabasal layers of the skin [71]. Depending on temperature and time, previtamin D3 is then isomerized to vitamin D. After binding to carrier proteins, in particular vitamin D-binding protein (DBP), vitamin D is transported to the liver where it is enzymatically hydroxylated by CYP27A1 (vitamin D-25-hydroxylase; 25OHD3) at the C25 position, generating 25-hydroxyvitamin D3 (calcidiol, 25OHD3). More recently, it has been found that all six cytochrome P450 isoforms (CYP27A1, CYP2R1, CYP2C11, CYP3A4, CYP2D25 and CYP2J3) exhibit vitamin D 25-hydroxylase activities [75, 76]. 25-Hydroxyvitamin D3, bound to DBP, is then transported to the kidney, and is finally hydroxylated by CYP27B1 (25-hydroxyvitamin D-1α-hydroxylase; 1αOHase) at C1α position to hormonally active calcitriol [1α,25-dihydroxyvitaminD3, 1α,25(OH)2D3]. Calcitriol acts in the kidney and is also transported by DBP to vitamin D receptor (VDR) positive target tissues (mainly bone, intestine and parathyroid gland) to act in a genomic or nongenomic manner. There is substantial evidence for additional extrarenal sites of calcitriol synthesis. In vitro, many nonrenal cells, including bone, placenta, prostate, keratinocytes, macrophages, T-lymphocytes and several cancer cells (e.g., from lung, prostate and skin) can enzymatically convert 25OHD3 to 1α,25(OH)2D3. A five-step inactivation pathway from calcitriol to calcitroic acid is attributed to a single multifunctional CYP, CYP24A1, which is transcriptionally induced by the action of calcitriol in a very sensitive manner. The physiological importance of a second catabolic pathway which includes the conversion of 1α,25(OH)2D3 to 1α,25(OH)2D3-3epi-D3 is less clear.

Skin cells (keratinocytes, fibroblasts and other cells) express VDR, an absolute prerequisite for regulation of genomic effects of calcitriol and other synthetic vitamin D analogues. Experimental and clinical findings have shown that the serum concentration of calcitriol (10⁻¹¹ to 10⁻¹⁰ M) is too low to induce VDR-mediated hormonal effects in the skin [77, 78]. More than 99% of the total circulating 1α,25(OH)2D3 is bound to carriers such as DBP and albumin. In the normal human only 0.4% of the circulating 1α,25(OH)2D3 is free [79]. According to the “free hor-
mone hypothesis" [80] only the free, and not total 1α,25(OH)₂D₃ regulates genomic processes within keratinocytes. This suggests that free plasma calcitriol approximates around 6×10⁻¹⁵ M. It has been shown in several studies that calcitriol, at concentrations higher than 10⁻⁸ M (equivalent to a highly unphysiological concentration of approximately 2.5×10⁻⁶ M total calcitriol in the circulating blood), is a potent growth inhibitor of normal human keratinocytes in vitro. In addition, cutaneous metabolism of circulating 25OHD₃ to 1α,25(OH)₂D₃ is suggested not to play a significant role in vivo because the amount of free 25OHD₃, which penetrates the cell membrane of epidermal keratinocytes is too small to induce formation of sufficient amounts of 1α,25(OH)₂D₃. 25OHD₃ is very tightly bound to DBP (Kₐ = 5×10⁻⁸ M) in circulating blood [81]. Due to this tight binding and the high plasma concentration of DBP (0.3 to 0.5 mg/ml), virtually all 25OHD₃ molecules in the circulation are present as a complex with DBP. Only approximately 0.03% of the metabolite is found in free form [82]. Furthermore, the deeper layers of the epidermis are not vascularized, which additionally impairs the passage of 25OHD₃ from blood to epidermal keratinocytes. Accordingly, no vitamin D₃ were found to serve as alternative substrates for 25OHD₃ [86]. Regulation of genes associated with growth, differentiation, apoptosis and other biological processes. There are a number of genes in keratinocytes which are primarily contributed to increased differentiation of keratinocytes in vitro and in vivo.

Recently, in vitro investigations have shown that dermal fibroblasts express one of the potential 25-hydroxylases (CYP27A1), but not the 1α-hydroxylase (CYP27B1). Therefore, fibroblasts might play an important role in the supply of calcitriol precursors (vitamin D₃ and 25OHD₃) to keratinocytes and possibly to circulating blood [89]. In recent studies with an in vitro system of reconstituted cytochrome P450 side-chain cleavage system (P450scct), 7-DHC and vitamin D₃ were found to serve as alternative substrates for P450scct [90]. Furthermore, it has been demonstrated that P450scct located in mitochondria from skin cells and other tissues can transform 7-DHC to 7-dehydroandrosterone (7-DHP) [91]. 7-DHP may serve as a substrate for further conversions into hydroxy derivatives through steroidogenic enzymes. In the skin, 5,7-steroidal dienes (7-DHP and its hydroxy derivatives) may undergo UVB-induced isomerization to vitamin D-like derivatives. This novel pathway can generate a variety of compounds depending on local steroidogenic activity and exposure to UVB. The physiological importance of this pathway remains, however, to be clarified.

It is commonly assumed that most of calcitriol formed by extra-renal cells serves an intracrine, autocrine or paracrine regulation within the cells in which it is produced. In particular, the epidermal keratinocyte is both: the site of calcitriol synthesis and target of this hormone. However, it remains to be shown whether and to what extent epidermal synthesis of calcitriol modulates cellular proliferation, differentiation, apoptosis, and immunological processes.

Gene regulatory effects of vitamin D₃ receptor in keratinocytes

1,25(OH)₂D₃ exerts its biological effects in keratinocytes and other cell types both via non-genomic and genomic mecha-
nisms. Genomic effects are mediated via binding to the nuclear receptor VDR which is the only nuclear protein that binds the hormone with high affinity (K_d = 0.1 nM) [92]. The VDR is one of 11 members of the nuclear receptor superfamily that exert their function as classical endocrine receptors. These include the already mentioned RARs, RXRs and the receptors for the nuclear hormones, thyroid hormone, estradiol, progesterone, testosterone, cortisol and aldosterone, which bind their specific ligand with a K_d of 1 nM or less [93]. Like most members of the nuclear receptor superfamily, the VDR contains two zinc finger structures that form a characteristic DNA-binding domain of 66 amino acids [94]. In addition, the carboxy-terminal of the protein contains a ligand-binding domain (LBD) of approximately 300 amino acids formed by 12 α-helices [95]. Ligand binding causes a conformational change within the LBD, whereby helix 12, the most carboxy-terminal α-helix, closes the ligand-binding pocket via a "mouse-trap like" intramolecular folding event [96]. The LBD is also involved in a variety of interactions with nuclear proteins, such as other members of the nuclear receptor superfamily, CoA and co-repressor proteins [97]. Co-repressor proteins, such as NCoR, SMRT and Alien, link non-liganded, DNA-bound VDR to enzymes with histone deacetylase activity that cause chromatin condensation [98]. This provides VDR with DNA-bound VDR to enzymes with histone deacetylase activity which cause chromatin condensation [98]. This provides VDR with intrinsic repressive properties comparable to retinoic acid and thyroid hormone receptors. The conformational change within VDR's LBD after binding of 1,25(OH)_2D3 results in the replacement of co-repressor molecules by a CoA protein of the p160-family, such as SRC-1, TIF2 and RAC3 [99], in complex with more general CoAs, such as CREB binding protein (CBP) [100]. These CoA complexes have histone acetyltransferase activity, whose action on their major nuclear substrate, histone N-terminus tails, have the net effect of causing chromatin relaxation [101]. In a subsequent step, ligand-activated VDR changes rapidly from a compact, closed conformation to a more extended conformation [96]. Ligand binding of 1,25(OH)_2D3 opens the LBD and alters its conformational potential [96].

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In recent years, 1,25(OH)_2D3 and its low-calcemic analogues have emerged as promising agents for the treatment of hyperproliferative diseases including psoriasis and cancer [110]. However, the mechanisms of the anti-proliferative, pro-differentiating and pro-apoptotic effects of VDR ligands vary and are cell-specific. They are mediated by the up-regulation of a broad variety of target genes, such as the cyclin-dependent kinase (CDK) inhibitors p21 and p27 [111] and the down-regulation of other target gene products including Bcl-2 [112] and Myc [113]. The p21(waf1/cip1) gene was suggested first by Jiang et al. [114] to be a ke gene for understanding the anti-proliferative action of 1,25(OH)_2D3. Moreover, the CYP24 gene was shown to be involved in vitamin D mediated growth control [115], since its expression significantly regulates 1,25(OH)_2D3 levels in keratinocytes and other cell types. The CYP24 gene is the most responsive human
primary VDR target gene and shows at the mRNA level up to 1000-fold inducibility by 1,25(OH)\(_2\)D\(_3\) [116]. Most other known primary 1,25(OH)\(_2\)D\(_3\) target genes, such as cyclin C and p21\(^{\text{waf1/cip1}}\), are much less responsive and often show an inducibility of 2-fold or less after short-term treatment with 1,25(OH)\(_2\)D\(_3\) [117, 118].

1,25-Dihydroxyvitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\), calcitriol] and analogues modulate epidermal proliferation and differentiation

Numerous in vitro and in vivo studies demonstrate dose-dependent effects of vitamin D analogues on proliferation and differentiation in keratinocytes and other cell types. At low concentrations, 1,25(OH)\(_2\)D\(_3\) stimulates proliferation of keratinocytes in vitro, while at higher pharmacological doses (≥ 10\(^{-8}\) M) keratinocyte proliferation is inhibited [119]. Consequently, vitamin D analogues are effective in the treatment of the hyperproliferative skin disease psoriasis. Immunohistochemical and biochemical analysis have demonstrated profound antiproliferative and differentiation-inducing effects in epidermal keratinocytes of lesional psoriatic skin along with treatment with vitamin D analogues in vivo [72, 73, 120, 121]. It has been shown that the immunohistochemical staining pattern for various markers of epidermal proliferation (e.g., proliferating cell nuclear antigen [PCNA], Ki-67-antigen) and differentiation (e.g., involucrin, transglutaminase K, filaggrin, cytokeratin 10) changes in lesional psoriatic skin along with topical treatment with vitamin D analogues almost completely to the staining pattern characteristic for nonlesional psoriatic or normal skin [120, 121]. Effects of topical treatment with vitamin D analogues on dermal inflammation in psoriatic skin are less pronounced. One reason for this observation may be that the bioavailability of this potent hormone in the dermal compartment may be markedly reduced as compared to the epidermal compartment [120]. Although the mechanisms that underlie the antiproliferative and differentiation-inducing effects of vitamin D analogues on keratinocytes are not completely understood, it is well known that these effects are at least in part genomic and mediated via VDR. It has been shown that keratinocytes from VDR-deficient mice do not respond to the antiproliferative effects of vitamin D analogues. The target genes of topical 1,25(OH)\(_2\)D\(_3\) that are responsible for its therapeutic efficacy in psoriasis are still unknown. Major candidates for 1,25(OH)\(_2\)D\(_3\) target genes that are responsible for the 1,25(OH)\(_2\)D\(_3\)-induced terminal differentiation in keratinocytes are distinct cell cycle associated proteins (e.g., INK4 family), including p21 [72, 111] and mitogens, such as insulin-like growth factors, that have also been reported to be down-regulated by 1,25(OH)\(_2\)D\(_3\) [122]. Recently, the binding factors for insulin-like growth factors (IGFBPs), which control the actions of mitogens, have been found to be primary mediators of the anti-proliferative actions of 1,25(OH)\(_2\)D\(_3\) in some cells, but dependent on cellular context, IGFBPs can also have a mitogenic effect. The IGFBP3 gene was shown to be a primary VDR target [123] and is therefore of special interest for understanding the mechanisms of the cell-regulatory actions of 1,25(OH)\(_2\)D\(_3\).

Immunomodulatory effects of 1,25(OH)\(_2\)D\(_3\) and analogues in the skin

During the last years, potent new immunomodulatory effects of vitamin D analogues have been characterized [124–126]. It has been demonstrated that various cell types involved in immunologic reactions (e.g., monocytes, T- and B-lymphocytes, Langerhans cells) do not only express VDR, but moreover possess the enzymatic machinery (25-hydroxyvitamin D\(_3\)-1α-hydroxylase) for the local synthesis of 1,25(OH)\(_2\)D\(_3\) [126]. Today, the local synthesis of 1,25(OH)\(_2\)D\(_3\) in immune cells is considered to be of critical importance for the regulation and control of immune responses. 1,25(OH)\(_2\)D\(_3\) inhibits activation of T-cells and induces the generation of CD25+/CD4+ regulatory T-cells [124]. In dendritic cells, 1,25(OH)\(_2\)D\(_3\) inhibits maturation and induces a phenotype that promotes tolerance and inhibits immunity after stimulation with antigen [124, 125]. Moreover, 1,25(OH)\(_2\)D\(_3\) suppresses expression of MHC II molecules and of co-stimulatory molecules including CD40, CD80 and CD86 in dendritic cells [126]. In these cells, production of interleukin (IL)-10 is stimulated and production of IL-12 inhibited, resulting in suppression of T-cell activation. At present, a connection between vitamin D and pathogenesis of atopic dermatitis is discussed. Epidemiologic studies have demonstrated that patients with atopic dermatitis have a lower vitamin D intake as compared to controls [127]. Additionally, it has been reported that vitamin D analogues suppress in vitro IgE-production and IgE-mediated cutaneous reactions [128, 129]. These immunomodulatory effects identify vitamin D analogues, most likely new vitamin D analogues with selective immunomodulatory activity, as promising new drugs for the prevention and therapy of inflammatory skin diseases including atopic dermatitis and allergic contact dermatitis [130].

1,25(OH)\(_2\)D\(_3\) and analogues as skin protecting agents

Recently, a new physiological function of the cutaneous photosynthesis of vitamin D has emerged: its putative role as an evo-
lutionary highly conserved hormonal system that protects the skin and other tissues against environmental hazards including ionizing and UV-radiation, microbial infections, and oxidative stress.

The hazardous effects of solar ultraviolet (UV) radiation, in particular of UVB with a wavelength range between 290 and 320 nm, represent the most important etiologic factor in the development of non-melanoma skin cancer [131, 132]. UVB induces photochemical changes in the skin that may lead to acute effects such as DNA-damage, sunburn and immune suppression or chronic effects like premature skin aging and skin cancer [131].

It has been demonstrated that 1,25(OH)2D3 protects human skin cells from UV-induced cell death and apoptosis [133, 134]. In these laboratory investigations, cytoprotective effects of 1,25(OH)2D3 on UVB-irradiated keratinocytes were seen morphologically and using a colorimetric cell survival assay [133]. Additionally, using an ELISA that detects DNA fragmentation, it was demonstrated that pretreatment with 1,25(OH)2D3 suppressed UVB-induced apoptotic cell death [133]. Pretreatment of keratinocytes with 1,25(OH)2D3 (1 μM) for 24 hours reduced UVB-stimulated apoptosis by 55–70%. Moreover, it was shown that pretreatment with 1,25(OH)2D3 also inhibited mitochondrial cytochrome c release (90%), a well known hallmark event of UVB-induced apoptosis [133]. Furthermore, it was demonstrated that 1,25(OH)2D3 suppressed two important mediators of the UV-response, namely, c-Jun NH2-terminal kinase (JNK) activation and IL-6 production [133]. Recent laboratory investigations have shown that pretreatment of keratinocytes with 1,25(OH)2D3 (1 μM for 24 h) efficiently inhibited UVB-induced PARP-cleavage [133]. Taken together, these findings indicate the existence of a photoprotective effect of active vitamin D analogues and open new perspectives for the potential pharmacological use of active vitamin D compounds in the prevention of UVB-induced skin damage and carcinogenesis [133–136]. Skin photocarcinogenesis is caused largely by DNA damage, most importantly mutations at sites of incorrectly repaired DNA photoproducts, of which the most common are the cyclobutane pyrimidine dimers (CPDs) [132]. It has been reported that 1,25(OH)2D3 protects primary human keratinocytes against the induction of CPDs by UVB [136]. These molecules may, therefore, represent promising candidates for the chemoprevention of UVB-induced skin cancer [136]. Additionally, topical application of 1,25(OH)2D3 reduces solar simulated UV-radiation (SSUV)-induced pyrimidine dimers in the epidermis of irradiated hairless Skh:HR1 mice [137]. Furthermore, UV-induced immunosuppression in the mice was markedly reduced by topical application of 1,25(OH)2D3 [137]. Altogether, these laboratory investigations convincingly demonstrate a protective effect of vitamin D compounds against UVB-induced photodamage in vitro and in vivo. It is tempting to speculate that the UVB-induced cutaneous production of vitamin D may represent an evolutionarily highly conserved hormonal feedback mechanism that protects the skin from the hazardous effects of solar UV-radiation.

The activation of the stress-activated protein kinases (SAPKs), such as c-Jun NH2-terminal kinase (JNK) and p38, represents an early cellular response to stress signals and an important determinant of cell fate. Modulation of these SAPKs is associated with cell death. The respective laboratory investigation has shown that the hormone also protected HaCaT keratinocytes from caspase-independent cell death induced by hyperosmotic or oxidative stresses [138]. The protection against hyperosmotic stress was not affected by inhibitors of the EGF receptor, ERK or PI3 kinase pathways, neither was it due to reduced activity of the proapoptotic p38 MAP kinase. In conclusion, these results are in agreement with previous in vivo observations that 1,25(OH)2D3 protects epidermal keratinocytes from apoptosis due to UV radiation or chemotherapy.

Recently, 1,25(OH)2D3 has been shown to represent a direct regulator of antimicrobial innate immune responses [140–142]. The innate immune system of mammals is of high importance providing a rapid response to repel assaults from numerous infectious agents including bacteria, viruses, fungi, and parasites. A major component of this system is a diverse combination of cationic antimicrobial peptides that are produced in various cell types, which include the α- and β-defensins and cathelicidins. Molecular mechanisms controlling the expression of CAMP are still poorly understood. Interestingly, the promoters of the human CAMP and defensin 2 (defB2) genes contain consensus VDRE that mediate 1,25(OH)2D3-dependent gene expression [141]. 1,25(OH)2D3 induces antimicrobial peptide gene expression in isolated human keratinocytes, monocytes and neutrophils, and human cell lines, and 1,25(OH)2D3 along with lipopolysaccharides synergistically induce CAMP expression in neutrophils [141]. Moreover, 1,25(OH)2D3 induces corresponding increases in antimicrobial proteins and secretion of antimicrobial activity against pathogens including Pseudomonas aeruginosa [140–142]. The induction of CAMP expression occurred via a consensus VDRE in the CAMP promoter that was bound by the VDR. In conclusion, there is convincing evidence that 1,25(OH)2D3 and its analogues directly regulate antimicrobial peptide gene expression in humans, revealing the potential of these compounds for the treatment of opportunistic infections.

**Future therapeutic applications of vitamin D3 and its analogues in skin diseases**

As outlined above, a broad variety of skin diseases including inflammatory skin diseases, infectious skin diseases and skin diseases with altered epidermal proliferation or differentiation represent promising targets for future therapeutic applications of vitamin D and its analogues. Besides studies to clarify the role of vitamin D analogues in the chemoprevention of cancer, clinical and laboratory studies are on their way to evaluate the safety and efficacy of several vitamin D analogues in the treatment of skin malignancies, including actinic keratoses and malignant...
melanoma [143]. At present, large efforts are made by the pharmaceutical industry to develop new vitamin D analogues that do not activate all vitamin D signaling pathways, but exert selective activity [143]. These new concepts for the development of new vitamin D analogues that exert selective activities (and most importantly reveal no or little systemic side effects) are based on recent progress in independent lines of investigation, demonstrating that vitamin D analogues may differ in their intracellular metabolism, nongenomic actions, pharmacokinetics, interaction with the DBP or the VDR [143].

Selective activity of vitamin D3 compounds via co-stimulation with synergistic acting drugs

Several in vitro and in vivo studies have shown that biological effects of 1,25(OH)2D3 can be selectively modulated by combination with various other drugs [143]. Interesting candidates to accelerate the effects of vitamin D compounds on cell proliferation and differentiation may be combination therapy of 1,25(OH)2D3 with ligands of nuclear VDR partner proteins, most importantly 9cRA, 1,25(OH)2D3 predominantly activates heterodimers of VDR and RXR, while in contrast stimulation 1,25(OH)2D3 alone induces preferentially the formation of VDR homodimers (Reichrath, unpublished data). In conclusion, combination therapy of 1,25(OH)2D3 with ligands of nuclear VDR partner proteins may selectively activate distinct classes of VDR dimers, thereby selectively modulating nuclear signaling pathways of vitamin D.

Tissue-selective potentiation of vitamin D3 activity via inhibition of vitamin D3 metabolizing enzymes

As explained above, 1,25(OH)2D3 levels are tightly controlled by its synthesis via the 1α-hydroxylase and its catabolism through hydroxylations mediated by specific cytochrome P-450 enzymes such as the 24-hydroxylase for 1,25(OH)2D3 [75]. Laboratory investigations have shown that a potential mechanism for the selectivity of vitamin D compounds is tissue-specific metabolism. Precedent for this has been demonstrated for other steroid hormone systems. Inhibition of 1,25(OH)2D3-catabolizing hydroxylation slows down catabolism and results in increased levels of 1,25(OH)2D3. Consequently, vitamin D activity in various target tissues can be potentiated by cytochrome P-450 enzyme-inhibiting drugs such as ketoconazole. Therefore, the expression of 1,25(OH)2D3-metabolizing cytochrome P-450 enzymes in target tissues is of high importance and deserves systematic analysis. Combination therapy of vitamin D analogues with inhibitors of vitamin D metabolizing enzymes may potentiate the biological effects of 1,25(OH)2D3 and analogues on cell proliferation and differentiation in target tissues that strongly express the 1,25(OH)2D3-metabolizing enzyme 24-hydroxylase without inducing substantial calcemic effects.

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