Vitamin A Antagonizes Decreased Cell Growth and Elevated Collagen-Degrading Matrix Metalloproteinases and Stimulates Collagen Accumulation in Naturally Aged Human Skin

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Damage to human skin due to ultraviolet light from the sun (photoaging) and damage occurring as a consequence of the passage of time (chronologic or natural aging) are considered to be distinct entities. Photoaging is caused in part by damage to skin connective tissue by increased elaboration of collagen-degrading matrix metalloproteinases, and by reduced collagen synthesis. As matrix metalloproteinase levels are known to rise in fibroblasts as a function of age, and as oxidant stress is believed to underlie changes associated with both photoaging and natural aging, we determined whether natural skin aging, like photoaging, gives rise to increased matrix metalloproteinases and reduced collagen synthesis. In addition, we determined whether topical vitamin A (retinol) could stimulate new collagen deposition in sun-protected aged skin, as it does in photoaged skin. Sun-protected skin samples were obtained from 72 individuals in four age groups: 18–29 y, 30–59 y, 60–79 y, and 80+ y. Histologic and cellular markers of connective tissue abnormalities were significantly elevated in the 60–79 y and 80+ y groups, compared with the two younger age groups. Increased matrix metalloproteinase levels and decreased collagen synthesis/expression were associated with this connective tissue damage. In a separate group of 53 individuals (80+ y of age), topical application of 1% vitamin A for 7 d increased fibroblast growth and collagen synthesis, and concomitantly reduced the levels of matrix-degrading matrix metalloproteinases. Our findings indicate that naturally aged, sun-protected skin and photoaged skin share important molecular features including connective tissue damage, elevated matrix metalloproteinase levels, and reduced collagen production. In addition, vitamin A treatment reduces matrix metalloproteinase expression and stimulates collagen synthesis in naturally aged, sun-protected skin, as it does in photoaged skin. Key words: fibroblast/gelatinase/interstitial collagenase/type I procollagen/type II procollagen. J Invest Dermatol 114:480–486, 2000

Skin becomes thin, dry, pale, and finely wrinkled with the passage of time (Smith et al, 1962; Lavker, 1979, 1995; West, 1994). In aging skin, the normal stages of epidermal differentiation are preserved, but epidermal thinning, associated with decreased numbers of keratinocytes, is observed histologically. The dermis also thins in aging skin, the result of reduction in the amount and organization of connective tissue (Smith et al, 1962; Lavker, 1979, 1995). Skin connective tissue is comprised primarily of fibrillar collagen bundles and elastic fibers, along with a complex array of proteoglycans and other extracellular matrix molecules. Dermal fibroblasts are imbedded within the matrix (Wenstrup et al, 1991). Collagen and elastin impart strength and resiliency to skin, and their degeneration with aging causes skin to become fragile, with easy bruising and loss of youthful appearance.

Ultraviolet (UV) irradiation from the sun damages human skin and causes premature skin aging (photoaging) (Kligman, 1969). Clinically, photoaged skin differs from sun-protected, naturally aged skin by having a thickened and rough appearance, with course wrinkles and mottled pigmentation. A hallmark of photoaged skin (not seen in sun-protected aged skin) is the presence of amorphous elastic material (Lavker, 1995). Damage to the collagen bundles that constitute the bulk (90% wet weight) of skin connective tissue is another prominent feature of photoaged skin. We have shown that UV irradiation induces synthesis of matrix metalloproteinases (MMP) in human skin in vivo (Fisher et al, 1996, 1997). We have proposed that MMP-mediated collagen destruction accounts, in large part, for the connective tissue damage that occurs in photoaging. In addition, we have reported that collagen synthesis is reduced in photoaged human skin (Griffiths et al, 1993; Talwar et al, 1995).

Traditionally, differences rather than similarities between naturally aged and photoaged skin have been emphasized (Smith et al, 1962; Lavker, 1979, 1995). We envisioned, however, that collagen damage in natural aging may arise, as it does in photoaging, from elevated MMP expression with a concomitant

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Histology and morphometry  Sections (5μm) from formalin-fixed skin samples were stained with hematoxylin and eosin and blinded. The number of cells present in the dermis of each section was determined, taking care not to include cells associated with epidermal structures or capillaries. In selected sections, it was determined that cells which were counted did not stain with antibodies to keratin (epithelial cells) or smooth muscle α-actin (myofibroblasts and smooth muscle cells). The cells thus characterized were operationally defined as fibroblasts, and this term will be used throughout the manuscript to describe the cells. The same sections were scored for four markers of connective tissue alteration: (i) fiber spacing; (ii) fiber thinness; (iii) fiber fragmentation; and (iv) depth of fiber fragmentation, using a scale of $1–9$ for each parameter.

Vehicle-treated and retinol-treated skin was also stained with a monoclonal antibody (MBI-1; Immunotech, Westbrook, ME) to the proliferation-associated antigen Ki-67 (Key et al., 1993).

Proliferation assays  Skin biopsies were cut into small fragments (15–20 fragments per biopsy) and each fragment placed in a well of 48-well dish. The fragments were incubated for up to 1 mo in Dulbecco’s modified minimal essential medium containing nonessential amino acids and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO$_2$. The number of tissue fragments from which fibroblasts were isolated (defined as spindle-shaped cells which were reactive with vimentin, but which did not stain with antibodies to keratin or with antibodies to smooth muscle α-actin) was determined, and expressed as a percentage of the total number of tissue fragments incubated. We have previously shown that isolation of fibroblasts from tissue fragments can be used as a reliable means for quantitating growth potential of fibroblasts within the tissue (Varani et al., 1994a, b).

MMP assays  Skin samples were frozen in liquid nitrogen immediately after collection, and kept frozen at −80°C until used for analyses. Skin samples were crushed under liquid nitrogen in mortar and pestle and homogenized in 20 mM Tris (pH 7.6), 5 mM CaCl$_2$. Insoluble material was removed by centrifugation and the supernatant used as the source of MMP. Collagenase enzyme levels were measured by hydrolysis of [H]$^3$H]labeled type I fibroblast collagen (Hu et al., 1978) after activation for 90 min with 1 mM aminophenyl mercaptoacetic acid. Western blot analysis with antibodies to interstitial collagenase (MMP-1) was performed as described (Fisher et al., 1996, 1997). Gelatinase levels (MMP-2; 72 kDa gelatinase and MMP-9; 92 kDa gelatinase) were measured by gelatin zymography (Mulligan et al., 1993) and quantitated by scanning laser densitometry. Although we routinely assessed total enzyme levels, active forms of the MMP were always present along with precursor forms. These could be seen in the western blots for MMP-1 and in the zymograms used to assess MMP-2 and MMP-9. Active enzyme forms ranged from less than 10% of the total in some specimens to greater than 75% in others. There were no consistent age-related differences in the percentage of enzyme in the active form. Likewise, there was no consistent effect of retinol treatment on the percentage of enzyme in the active form.

Collagen synthesis  Type I procollagen (α1 chain) protein levels were assessed by western blot analysis and by immunohistology as described (Talwar et al., 1995). Type III procollagen immunohistology (α1 chain) was performed as described (Griffiths et al., 1993) using an antibody from Chemicon International (Temecula, CA). Total collagen biosynthesis by fresh skin samples was assessed by incorporation of [14C]proline into pepsin-resistant, trichloroacetic acid (TCA)–precipitable material, as described previously (Sykes 1976, Sykes et al., 1976, Varani et al., 1990). Skin samples that had been freeze-thawed prior to incubation with [14C]proline (to disrupt cells and thereby prevent collagen biosynthesis) served as a control for non-specific label incorporation. To measure type I procollagen biosynthesis specifically, fresh skin samples were incubated for 24 h in keratinocyte basal medium (Clonetics, Walkersville, MD), supplemented with Ca$^{2+}$ to a final concentration of 1.4 mM. At the end of the incubation period, media were collected and analyzed for type I procollagen protein by enzyme-linked immunosorbent assay (ELISA) (PanVera, Madison, WI).

In situ detection of type I procollagen (α1) mRNA  Type I procollagen (α1) gene expression in skin specimens was assessed by in situ hybridization. Frozen sections were hybridized with digoxigenin-labeled anti-sense and sense type I procollagen α1 cRNA probes as described previously (Kang et al., 1995).

Detection of type III procollagen (α1) mRNA by reverse transcription–polymerase chain reaction (reverse transcription–PCR)  Reverse transcription–PCR was used to assess type III procollagen gene expression. Total RNA was isolated from skin.
Figure 2. Histologic features of skin connective tissue are altered in aged skin. Representative histology of skin connective tissue seen in sun-protected skin from a young person (22 y old; a, b) and from 7 d vehicle-treated (c, d) and retinol-treated (e, f) skin from an aged person (86 y old). Formalin-fixed skin sections were stained with hematoxylin and eosin. Scale bar: (a, c, e) 100 μm; (b, d, f) 50 μm.

Figure 3. Fibroblast growth potential is reduced with increasing age and is increased with retinol treatment. Freshly obtained skin specimens from persons of varying age were cut into small pieces (15–20 pieces/skin sample) and placed in culture to allow outgrowth of fibroblasts from the tissue. Skin from persons in the four age groups (n = 12, 11, 12, 10, respectively) was analyzed. Data are presented as the percentage of skin pieces from which fibroblasts were isolated. *p < 0.05 versus 18–29 y old group. Fibroblast outgrowth was also determined in skin samples from 17 persons 80+ y of age who were treated with 1% retinol and its vehicle for 7 d. *p < 0.05 versus vehicle-treated skin. Values are mean ± SEM.

Figure 4. MMP levels are increased in skin with increasing age and partially reduced with retinol treatment. Skin samples of persons of varying age were analyzed for expression of three MMP: (a) MMP-1 (interstitial collagenase) (n = 10 persons per age group); (b) MMP-9 (92 kDa gelatinsase); and (c) MMP-2 (72 kDa gelatinsase) (n = 10, 11, 8, and 8 persons per age group). Values are mean ± SEM. *p < 0.05 versus 18–29 y old group. ***p < 0.01 versus 18–29 y old age group. **p < 0.01 versus 18–29 y old age group. Skin samples from 16 persons 80+ y of age who were treated with 1% retinol and vehicle for 7 d were also analyzed for the three MMP. Values are mean ± SEM. Values for the vehicle-treated 80+ y old individuals were normalized to 1.0 and the other values expressed relative to the normalized values. ***p < 0.001, retinol versus vehicle-treated skin.

RESULTS

Connective tissue alterations increase with increasing age. We assessed the degree of connective tissue damage in sun-protected skin from individuals in each of four age groups (18–29, 30–59, 60–79, and 80+ y). With increasing age, there was progressive loss of dermal fibroblasts (Fig 1b), and increased dermal connective tissue abnormalities, as indicated by increased space between connective tissue fiber bundles, increased thinning of connective tissue fiber bundles, increased disorganization of fiber

Statistical analysis. Data were evaluated using analysis of variance followed by paired group comparisons for studies involving individuals in different age groups, and using paired t tests for comparisons between vehicle-treated and retinol-treated skin. All differences were two-tailed (Woodson, 1987). p < 0.05 were considered statistically different.
bundles and increased depth to which disorganization extended (Fig. 1b). Dermal cellularity and connective tissue features were similar in the two youngest age groups (18–29 and 30–59). Persons aged 60–79 and 80+ y had significantly reduced dermal cellularity and increased connective tissue abnormalities, compared with persons 18–29 y (p < 0.05 for both parameters in the 60–79 y old group and p < 0.001 and 0.05 for the same parameters in the 80+ y old group). Topical treatment of sun-protected skin of 80+ y old individuals with retinol for 7 d increased dermal cellularity approximately 25% (p = 0.009; n = 17) (Fig. 1a). Staining of vehicle-treated and retinol-treated skin from 80+ y old individuals with a monoclonal antibody to the proliferation-associated antigen, Ki-67, revealed a significantly higher number of reactive cells in the retinol-treated skin than in skin treated with vehicle alone (20 ± 3 cells in retinol-treated skin versus 3 ± 1 cells in vehicle-treated skin (p < 0.01; n = 5). Although retinol treatment for as little as 7 d induced measurable changes in the dermal fibroblast population, this short-term retinol treatment did not alter age-associated connective tissue abnormalities (Fig. 1b). Figure 2 shows the typical histologic appearance of dermal connective tissue in sun-protected skin of a 22 y old individual, and vehicle-treated and retinol-treated sun-protected skin of an 86 y old individual. Reduced numbers of fibroblasts and alterations in connective tissue structure are apparent in the aged (vehicle-treated) skin relative to the young skin. Also apparent is the increased number of fibroblasts in the aged retinol-treated skin.

**Fibroblast growth potential decreases with increasing age and is stimulated with retinol** Fibroblast outgrowth from skin fragments was used as a measure of fibroblast growth potential within the tissue (Fig. 3). Fibroblast outgrowth declined with increasing age. In the 18–29 y old group, fibroblasts were isolated from 182 of 240 tissue fragments (n = 12 subjects, 76%). The percentage of tissue fragments from which fibroblasts were obtained decreased with donor age until in the 80+ y old group, fibroblasts were isolated from only 76 of 200 tissue fragments (n = 10 subjects, 38%) (p < 0.05 compared with the 18–29 y old group). Treatment of 80+ y old individuals with retinol for 7 d increased fibroblast outgrowth greater than 3-fold (46 of 255 tissue fragments or 18% in the vehicle-treated group versus 144 of 255 tissue fragments or 56% in the retinol-treated group) (p < 0.05; n = 17).

**MMP levels increase with increasing age and are reduced by retinol** We next assessed levels of three connective tissue-degrading MMP, including MMP-1 (interstitial collagenase), MMP-9 (92 kDa gelatinase) and MMP-2 (72 kDa gelatinase), in sun-protected skin as a function of age. All three MMP were elevated in the 80+ y old group, compared with the 18–29 y old group (approximately 40, 52, and 82% for MMP-1, MMP-9, and MMP-2, respectively) (p < 0.01, 0.05, and 0.001) (Fig. 4). The three MMP were also elevated in the 60–79 y old group (23, 20, and 44%, respectively). Western blot analysis performed on skin samples from a separate group of 18–29 and 80+ y old individuals revealed that MMP-1 protein levels were increased by approximately 40% in skin samples from persons 80+ y old (p < 0.02, n = 16), compared with persons 18–29 y of age (Fig. 5).

**MMP-1, MMP-2, and MMP-9 levels were also assessed in retinol-treated and vehicle-treated skin from 80+ y old individuals. Retinol treatment reduced MMP-1 and MMP-9 levels to levels seen in persons 18–29 y old (p < 0.001; n = 16). In contrast, retinol treatment had no effect on the elevated MMP-2 level in skin of persons in the 80+ y old group (Fig. 4).**

**Type I and type III procollagen expression are decreased in aged skin** Type I procollagen (α1 chain) protein levels were assessed by western blot analysis in skin samples from persons 18–29 y of age and 80+ y of age. Type I procollagen expression was decreased by 52% in aged skin, compared with skin from younger persons (n = 16, p = 0.022) (Fig. 6a). Immunohistochemistry of type I procollagen revealed prominent extracellular staining in the dermis, adjacent to the dermoepidermal junction, in skin of persons 18–29 y of age. This staining was substantially reduced in skin of persons 80+ y of age (Fig. 6b). We also performed immunohistochemistry of type III procollagen (α1 chain). In skin from young persons, type III procollagen was found associated with...
mature collagen fibers throughout the dermis. This dermal staining was considerably reduced in the skin of aged persons (Fig 6b).

**Collagen synthesis in skin of 80+ y old individuals is stimulated by retinol** As described above, sun-protected skin of persons in the 80+ y old age group contains reduced numbers of fibroblasts, with diminished growth potential, increased MMP levels, and reduced expression of type I and III procollagen. These properties would be expected to cause a deficit in connective tissue collagen. The finding that 7 d retinol treatment substantially restored fibroblast numbers and growth potential, and reduced MMP levels suggests that retinol might increase collagen content of aged skin. We therefore assessed collagen biosynthesis in vehicle-treated and retinol-treated skin from 80+ y old individuals by three methods: (i) ex vivo incorporation of [14C]proline into total collagen; (ii) ex vivo secretion of type I procollagen protein; and (iii) in vivo expression of type I and type III procollagen mRNA. As shown in Fig 7(a), collagen biosynthesis, measured by [14C]proline incorporation, was increased 1.4-fold in skin treated with retinol relative to skin treated with vehicle (p = 0.03; n = 9). Analysis of the radioactive collagen fraction by sodium dodecylsulfate-polyacrylamide gel electrophoresis revealed that the α1 and α2 chains of type I collagen accounted for the majority of the [14C]proline incorporation (not shown).

We also utilized an ELISA procedure to measure production of type I procollagen by retinol-treated and vehicle-treated skin samples during a 24 h period. Skin samples from 80+ y old persons that had been treated in vivo with retinol for 7 d produced 65% more type I procollagen than matched vehicle-treated skin (p < 0.05; n = 9) (Fig 7b). This increase is consistent with the increase in collagen synthesis measured by [14C]proline incorporation (Fig 7a).

In addition, we examined type I procollagen (α1 chain) gene expression by in situ hybridization in retinol-treated and vehicle-treated skin of 80+ y old persons. The number of cells expressing detectable levels of type I procollagen mRNA was approximately

Figure 7. Retinol treatment increases collagen synthesis in aged (80 + y) skin. (a) Freshly obtained skin samples from nine persons treated with 1% retinol and its vehicle for 7 d were incubated for 24 h with [14C]proline. The amount of radioactivity (counts per minute, cpm) incorporated into the collagen fraction was determined. Values are mean ± SEM. *p < 0.05 versus vehicle-treated skin. (b) Freshly obtained skin samples from nine persons treated with 1% retinol and its vehicle for 7 d were incubated in culture medium for 24 h. The amount of type I procollagen secreted into the medium was determined by ELISA. Values from vehicle-treated skin were assigned a number of 1.0 and the values from corresponding retinol-treated skin normalized to this value. Values are mean ± SEM. *p < 0.05 versus vehicle-treated skin. (c) Type I procollagen α1 gene expression was measured by in situ hybridization in frozen skin sections from seven persons treated with 1% retinol and its vehicle for 7 d. The number of cells expressing detectable type I procollagen α1 mRNA in three sections from each skin sample were counted. **p < 0.01 versus vehicle-treated skin. (Insert: Vehicle-treated and retinol-treated skin from an 87 y old person; scale bar: 150 μm). (d) Type III procollagen α1 gene expression was measured in skin samples from seven persons treated with 1% retinol and its vehicle for 7 d by reverse transcription-PCR. The values of the vehicle-treated skin were assigned a number of 1.0, and values from corresponding retinol-treated skin normalized to this value. Values are mean ± SEM. **p < 0.01 versus vehicle-treated skin.
2.8-fold higher in retinol-treated skin, compared with vehicle-treated skin (p = 0.003; n = 7) (Fig 7c). The majority of fibroblasts that were positive for type I procollagen mRNA were in the upper dermis, immediately below the dermoepidermal junction (Fig 7f, insert). Consistent with these findings, type I procollagen was detectable in the dermal fibroblasts from retinol-treated skin, whereas staining for type I procollagen was not evident in vehicle-treated tissue from the same individuals (Fig 8). Finally, we utilized semiquantitative reverse transcription–PCR to assess type III procollagen mRNA levels in retinol-treated and vehicle-treated skin of 80+ y old persons. Type III procollagen mRNA levels were 5-fold higher in retinol-treated skin samples, compared with vehicle-treated skin (p = 0.0015; n = 7) (Fig 7d).

**DISCUSSION**

A number of changes occur in the structure of skin connective tissue as a consequence of the natural aging process. Age-related changes include a decrease in the number of interstitial fibroblasts, a thinning of connective tissue (collagen) fiber bundles, an increase in space between connective tissue fiber bundles, and an increase in histologically observable connective tissue disorganization. These changes are evident in many of the individuals between the ages of 60 and 79 y, and are apparent in virtually every individual 80 y or older. It is thought that these alterations in the dermal connective tissue are largely responsible for the thin, fragile, and finely wrinkled quality of naturally aged skin (Lovell et al., 1987; Miyahara et al., 1992).

What accounts for these changes in the structure of sun-protected skin during chronologic aging? This study provides direct evidence that there is reduced fibroblast proliferation (as indicated in the ex vivo growth assay), increased MMP expression (as assessed by collagen degradation, gelatin zymography, and western blotting) and reduced elaboration of new collagen (mRNA and protein). Previous studies using cells in monolayer cultures have demonstrated reduced proliferative capacity of dermal fibroblasts with age (Plisko and Gilchrest, 1983; Gilchrest, 1983; Stanulis-Prager and Gilchrest, 1986; Sauder et al., 1988). Other in vitro studies have shown that MMP expression by skin cells increases as they age (Millis et al., 1989, 1992; West et al., 1989; Burke et al., 1994; Bizot-Foulon et al., 1995; Ricciarelli et al., 1999), and still other in vitro studies have demonstrated decreased collagen synthesis in aged fibroblasts (Johnson et al., 1986; Gregory et al., 1986; Mays et al., 1990; Furth, 1991).

This study also demonstrates that these changes observed in aged skin may be partially reversed by treatment with topical retinol (vitamin A). Treatment of aged, sun-protected skin with 1% retinol for only 7 d resulted in increased numbers of fibroblasts in the skin. In parallel, topical treatment with 1% retinol for 7 d increased fibroblast growth from tissue specimens, reduced collagenase, and gelatinase expression in these same tissues and stimulated new collagen synthesis.

How retinol acts to reverse age-associated changes in aged skin in vivo is not fully understood. With regard to MMP expression, we recently demonstrated that UV irradiation upregulates transcription factor AP-1 and its target genes, MMP-1 and MMP-9, in human skin in vivo (Fisher et al., 1996, 1997). MMP-2 gene expression is not regulated by AP-1 and is not induced by UV in human skin. All-trans retinoic acid, which is formed from retinol in human skin (Kang et al., 1995), was found to inhibit AP-1 activation as well as MMP-1 and MMP-9 gene expression (Fisher et al., 1996, 1997). Expression of MMP-2 was not inhibited by all-trans retinoic acid in photoaged skin (Fisher et al., 1996), nor by retinol in naturally aged skin (this study), consistent with the fact that its promoter lacks an AP-1 site. Elevated levels of MMP-1 and MMP-9 in aged skin in vivo and the reduction in both activities by retinol are consistent with AP-1 activation during aging and with retinol repression of AP-1 activation. In photoaged skin, oxidant stress is thought to play a part in the signaling events that lead to MMP upregulation (Brennenseis et al., 1998). Oxidant stress in the natural aging process (Sohal and Weindruch, 1996) may, likewise, lead to MMP induction.

MMP inhibition by topical retinol could suppress degradation of newly synthesized procollagen, and thereby lead to enhanced procollagen expression. Alternatively, new collagen biosynthesis may be directly stimulated by retinol. Whereas [14C]proline incorporation experiments and ELISA results cannot distinguish between decreased breakdown and increased synthesis of collagen, the existence of elevated mRNAs for both type I and type III procollagen in retinol-treated skin strongly argues that new collagen synthesis is at least partially responsible for the enhanced procollagen levels. This increased procollagen production could occur as a result of fibroblast activation. Past studies have shown that treatment of human skin in organ culture with all-trans retinoic acid increases overall protein synthesis and the production of several components of the extracellular matrix (Varani et al., 1993, 1994a). As another possibility, retinol-induced proliferation of fibroblasts in aged skin could result in increased collagen production simply as a secondary consequence of the presence of additional collagen-producing cells. Ultimately, these possibilities are not mutually exclusive.

The findings presented here are of interest from a number of standpoints. First, they indicate that there is significant overlap in the pathophysiology of natural skin aging and sun-induced premature skin aging (photoaging), although the etiologies are different. Equally important, our findings suggest that naturally aged, sun-protected skin and photoaged skin respond to topical retinoid treatment in an analogous manner. It has been established that topical retinoid treatment can partially reverse some of the clinical and histologic features associated with sun-induced premature skin aging (Kligman et al., 1986; Weiss et al., 1988). Recently, it has been shown in a small, clinical study that topical retinoid treatment can also improve the clinical appearance of aged, sun-protected skin. Although epidermal changes were the major focus of this study, cytologic evidence of fibroblast activation in the retinoid-treated skin was noted (Kligman et al., 1993). The ability of retinoids to induce the repair of connective tissue damage in naturally aged skin suggests that topical retinoid treatment might be useful for treating and preventing the thin, fragile skin of aged persons. If this proves to be the case, retinol (and other retinoids) may be beneficial for long-term use in aged populations. This will, of course, need to be established in controlled clinical studies where drug dosage, formulation, and application conditions are all carefully considered and evaluated.

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