Immunohistochemical Analysis of Steroid Hormone Receptors and Structural Proteins in Skin: Effects of Aging and Hormone Status

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ABSTRACT

Reports describing beneficial effects of estrogens on skin structure suggest that skin may contain estrogen receptors (ERs). The present study was designed to determine the presence of these receptors and related proteins in skin from the dorsal forearm and buttocks of 12 premenopausal and 13 postmenopausal subjects who were not taking any hormonal supplements. Fixed, sectioned samples were stained with hematoxylin and eosin for general characterization with Verhoeff-van Gieson elastic stain, and with immunohistochemical staining for ERs, progesterone receptors, and fibronectin. The results showed that fibronectin and elastin fibers were present and were more abundant in sun-exposed skin. In contrast, ERs and progesterone receptors were not found in skin. The absence of ERs and progesterone receptors in all samples weakens the hypothesis that estrogen exerts direct receptor-mediated actions on skin.

INTRODUCTION

The possible role of estrogens in the maintenance and healing of normal skin is suggested by studies using animal models, human cells in culture, and human subjects. Estrogen enhanced both the rate and the extent of wound healing in rat skin,[1] and ovariectomy rendered rats more susceptible to photoaging.[2] Rudolph and Vande Berg[3] found that fibroblasts from sun-exposed skin of women receiving hormone replacement therapy (HRT) survived through significantly more cycles of replication before senescence in vitro than did similar cells from women not receiving HRT. They concluded that survival of sun-exposed skin might be improved by use of HRT in postmenopausal women.

The effective reduction in estrogen following menopause has been proposed as a cause of accelerated aging of human skin. Affinito et al[4] found that the collagen content as well as the ratio of type III to type I collagen in the skin showed a strong inverse correlation with years postmenopausal, irrespective of age at menopause. This suggests that the accelerated collagen decrease is

related to estrogen loss, rather than due to aging per se.

A more direct approach to investigating the role of estrogen was performed by examining the effects of HRT on physical and mechanical properties of skin in postmenopausal women. Several reports indicate that the decrease in collagen content of human skin after menopause was diminished or abolished by use of systemic HRT,[5-7] particularly for type III collagen.[8] HRT also was found to maintain the mechanical properties of skin[9] and to prevent postmenopausal atrophy of skin as measured by overall thickness.[10] Sator et al[11] observed improved skin moisture, elasticity, and thickness in postmenopausal subjects receiving 6 months of estrogen replacement therapy. These studies suggest a role for estrogens in the preservation of normal skin structure.

However, not all studies have reported positive results. Henry et al[12] found that while HRT prevented rheological changes in skin related to aging, it did not alter the development of fine wrinkles in facial skin. Similar findings were reported by Castelo-Branco et al,[13] who found that smoking greatly increased facial wrinkling in postmenopausal women, and that HRT did not have a significant effect on this process. Despite these reports, most evidence suggests that estrogens do have a protective effect on the aging of skin.

The cited studies did not specifically address the mechanism of action of hormones in controlling the skin structure. The effects of HRT on skin structure and aging may be related to classical receptor-mediated responses. This would allow for the efficacy of topical application of estrogens to the skin, while avoiding the potential for adverse effects now associated with systemic HRT.

To test this assumption, we performed a detailed examination of the presence of specific receptors for estrogen and progesterone in the skin of both premenopausal and postmenopausal women. In addition, we performed a qualitative and quantitative analysis of key structural proteins in the skin, assessed the general histology and morphology of the skin, and noted evidence of sun exposure in the skin. Our findings provide insight into the roles of estrogen and related hormones in skin aging.

METHODS AND MATERIALS

All subjects were healthy nonsmokers who were within 20% of their ideal body weight and had Fitzpatrick skin pigment types of III or less. Premenopausal subjects ranged in age from 26 to 35 years, were not pregnant or nursing and had not taken oral contraceptives for at least 2 years. Examinations and biopsies were scheduled during the first week of their menstrual cycle in order to standardize their hormonal status. Postmenopausal subjects ranged in age from 46 to 58 years and had not received HRT for at least 3 years.

The subjects were examined by a dermatologist and blood was drawn for determination of follicle stimulating hormone and luteinizing hormone levels. Two 4-mm punch biopsies were performed: one from the right dorsal forearm, 1 cm inferior to the elbow crease; and the second from the inferior aspect of the right buttocks, 8 cm lateral to the midline. All procedures and documents were reviewed and approved by the Human Subjects Committee of the University of Kansas Medical Center.

Tissue specimens from sun-exposed (ie, forearm) and nonexposed areas (ie, buttocks) were fixed in formalin and embedded together in paraffin blocks. They were subsequently sectioned and stained with hematoxylin and eosin for histopathologic evaluation. Verhoeff-van

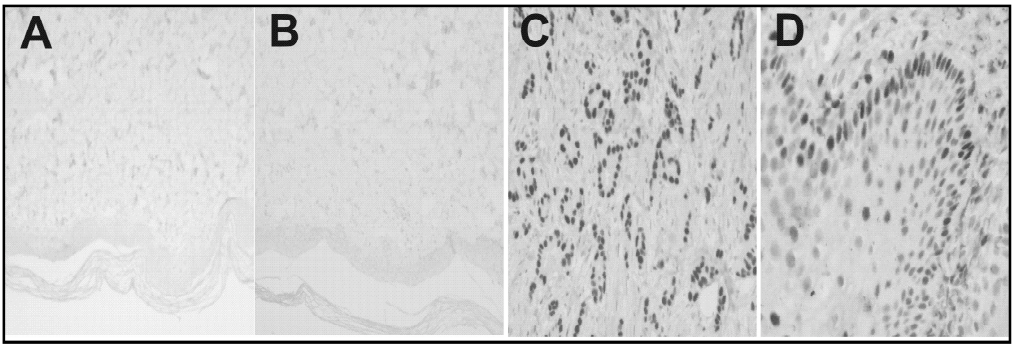

Figure 1. Immunohistochemical Staining for ER-β in Human Skin and Tissues. A) dorsal forearm (sun-exposed); B) buttocks (non–sun-exposed); C) breast carcinoma; D) cervix.

Gieson elastic stain was used to study elastic fiber fragmentation. Immunohistochemical staining was carried out on a DAKO autostainer using the DAKO LSAB+ detection system. Monoclonal antibodies were directed against estrogen and progesterone receptors (ERs and PRs) and fibronectin (DAKO, Carpentira CA, 1:400, 1:2000, and 1:5000, respectively). The sections were deparaffinized in xylene and rehydrated in alcohol, followed by blockage of endogenous peroxidase by incubation with 0.3% hydrogen peroxide for 30 minutes. Antigen retrieval was performed with 10-mM citrate buffer. The primary antibody was applied to sections for 15 to 30 minutes, followed by incubation with biotinylated anti-immunoglobulin and diaminobenzidine-hydrogen peroxide. Methyl green was used as a counterstain. A breast carcinoma section that we routinely use as a positive control for ERs and PRs was processed in parallel. In addition, a human cervical biopsy specimen from the Department of Pathology section library, immunostained for ER-β, was included for comparison with the experimental samples.

RESULTS

We recruited 12 premenopausal and two postmenopausal subjects at the University of Kansas Medical Center. Avon Products Research Group provided biopsy specimens from an additional 11 postmenopausal subjects. Follicle-stimulating hormone and luteinizing hormone levels confirmed that all premenopausal subjects were in the follicular phase of their cycle, and gave appropriate values for the postmenopausal subjects.

Sections from each biopsy were analyzed in duplicate for each staining procedure. Hematoxylin and eosin staining demonstrated basophilic degeneration of collagen in the sun-exposed skin. Despite thorough attempts with two different antibodies against ER-β this antigen was not detected in skin sections. In contrast, ER-β was easily detected in the positive controls (Figure 1, panels C and D), but was not detected in any of the biopsy specimens. Representative sections from sun-exposed and nonexposed skin of a premenopausal subject are shown in Figure 1, panels A and B, respectively. Panel 1D is from a human cervical biopsy, and clearly shows strongly positive staining for ER-β in squamous epithelium, but not in surrounding cells. Identical results were obtained for PRs, in that this antigen was clearly demonstrated in the same breast carcinoma, but was undetectable in all skin samples (data not shown). All

subjects except one showed a marked increase in fibronectin expression in the sun-exposed skin compared with nonexposed skin. In addition, sun-exposed skin also showed an increase in elastin fibers.

DISCUSSION

The absence of detectable ERs in human skin found in this study was surprising, given the findings of previous studies. Hasselquist et al[14] performed binding studies using homogenates of human tissues and reported high-affinity estradiol binding sites in homogenates from skin and uterus tissue. This experimental approach, however, did not allow for the unequivocal identification of ERs, nor did it yield information regarding cell type or subcellular localization of binding sites. Also, the number of binding sites differed substantially depending on the anatomical source of the skin. Facial skin exhibited the highest binding levels, approximately double that of breast skin and over three times the level observed in skin of the thigh. Even for facial skin, the binding density was only 5% the level of estradiol binding in uterus. This extremely low level of binding sites questions the biological relevance of these sites.

An extensive immunohistochemical analysis for p29—proposed as a marker of ERs—in skin was reported by Jemec and Wojnarowska.[15] All samples were positive for the presence of p29, regardless of subject age or gender or anatomical site of skin sample. Although the authors equated the presence of p29 with that of ER, this association may be spurious. Vargas et al[16] found expression of p29 in 109 of 111 human cell lung carcinoma samples despite the complete absence of ERs in any of the tissues, severely weakening this hypothesized correlation between p29 and ER expression.

Additional immunohistochemical studies of ERs in human skin have provided mixed results. Hodgins et al[17] found ERs and PRs in the epidermis of the labia minora, but only in basal keratinocytes of true skin. Furthermore, a clear gradient of diminishing expression for these receptors was found in the transition from vaginal epithelium—where the highest levels were observed—to labial epidermis. They concluded that, due to this low level of expression of ERs, direct effects of estrogens were unlikely in the vulva.

Similar results were found in an examination of primary mucinous carcinomas of the skin.[18] Although ERs and PRs were present in these tumors, their presence correlated to the mammary lineage of the tumor rather than to characteristics of true skin. Offidani and Campanati[19] found that ERs and PRs were highly expressed in female anogenital sweat glands, and proposed this as a distinguishing feature for differentiating between those glands and conventional sweat glands. Together, these results suggest that expression of ERs and PRs in skin is a reflection of specialized, sexually dimorphic sites rather than a general feature of skin, a conclusion shared by Gunes and Fetil.[20]

Recent reports state that ER-β is found in human abdominal[21] and nonbalding scalp skin.[22] The scalp showed ER-β in the keratinocytes of the stratum basale and stratum spinosum, hair follicle bulb, and in sebocytes of sebaceous glands.[22] In addition, female abdominal skin exhibited extensive labeling of keratinocytes.[21] The difference between these positive findings and the negative findings in the present study may reflect anatomical and functional differences in ER-β expression in skin, rather than differences in study methodology (ie, specific antibodies or procedures employed). In particular, ER-β is described as most apparent in areas of skin with high hair follicle density,[22] which are unlike the anatomical sites

chosen for this study. This association of ERs with high density of follicles may be key to the differential anatomical expression of this receptor.

The apparent contradiction between the observed effects of estrogens on the appearance and structure of human skin and the lack of detectable ERs found in this study could have several possible explanations. First, the receptors may actually be present, but at levels too low for detection. A previous study reported that the levels of high affinity estrogen binding sites in human skin homogenates were at least 20-fold lower than the levels found in known estrogen-responsive organs and tissues, and that the concentration of binding sites varied dramatically depending on the anatomical source of the skin.[14] It may be that levels of ER detectable by immunohistochemical techniques, and which might be present at biologically relevant levels, could be found in skin from the face or other areas that were not examined in the present study.

A second possibility is that estrogens do have a direct effect on skin, but that this effect is not dependent on the classical ER mechanism. Indeed, estrogens are known to have effects in certain cells and tissues that are far too rapid to occur via receptor activation and altered gene expression.[23] These estrogen responses have been proposed to involve membrane-bound forms of ER that might not be recognized by antibodies against soluble ER and which may modulate G-protein coupling pathways.

A less direct effect of estrogens on skin has been proposed whereby the target cells for hormone action are not skin cells. Studies of estrogen effects on wound healing found that the beneficial effects of topical estrogen were primarily the result of effects on infiltrating neutrophils, and specifically due to inhibition of neutrophil chemotaxis.[1,24] This finding is bolstered by the immunohistochemical analysis of ERs and PRs in human vulva and vagina by Hodgins et al.[17] They observed scant evidence for ERs and no evidence for PRs in true skin. Moreover, they concluded that modulation of immunologic and inflammatory systems was the main effect of estrogens on skin.

A final explanation could be that the major influence of estrogens on the skin may result from a systemic effect, which would not depend on any form of ER in the skin.[17] Of particular note, the majority of published reports on the effects of estrogens on skin involved oral administration of hormones, which influence a wide range of physiological systems. Even in those studies that did involve topical application of estrogens as creams or patches, the question of local versus systemic effects still may be raised.

Absorption of estrogens for systemic effects following dermal application is well known. For example, Schmidt et al[25] found a modest increase in plasma estradiol levels in postmenopausal subjects receiving a cream containing 0.01% estradiol; however, the increase was not statistically significant. Although other studies did not measure the effects of topical treatments on systemic estrogen levels, some experimental designs have allowed for comparison between local and systemic effects of topical estrogens.

Callens et al[10] applied an estradiol-containing gel or patch to the skin and then measured skin thickness and sebum at the site of the patch and at four other sites. Significant effects on skin thickness were observed at the site of application, denoting a local effect, and also in the skin of the breast, which could result from systemic effects of estradiol. Interestingly, no effect was seen at the other sites examined (ie, forearm and forehead). These findings suggest that

although the major effect of topical estrogens is at the site of application, effects at a remote site can result from absorption and distribution of the hormone.

In conclusion, extensive anecdotal evidence coupled with the findings from more rigorous clinical and laboratory investigations support a role for estrogens in the maintenance of the structure of human skin, protection of the skin from aging and photoaging, and healing following injury. We have examined the presence of ERs in human skin from two anatomical sites. These receptors are the key components required for the simplest, most direct mechanism of estrogen action in skin. Our inability to detect ERs in these skin specimens, although tempered by the inability to validate ER-□ detection in human tissues, nevertheless suggests that either there is extensive anatomical variation in the characteristics and regulation of skin structure and function, or there are nonclassical mechanisms for estrogen effects on this tissue.

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