SKIN IMMMUNOSENESCENCE: THE CRITICAL INVOLVEMENT OF HORMONAL BALANCE AFFECTING SIGNAL TRANSDUCTION

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Abstract: *Objectives of review:* This manuscript aims to review the state of the art on skin aging with particular attention on skin immunosenescence, hormonal changes and signal transduction. *Recent findings:* Skin immunosenescence accounts, in the elderly, for increased susceptibility to cutaneous infections and malignancies, and decreased response to vaccination. Many factors have been proposed to contribute to immunosenescence. We recently described an aging-related decrease in receptor for activated C kinase (RACK)-1 expression, defective protein kinease Cβ II (PKC) translocation and reduced TNF-α release in rat epidermal cells. Given the observation that adrenal hormones follow a decrease during aging, our group has also demonstrated that the decreased level of RACK-1 protein expression during aging and the observed immune deficits in rats could be restored in vivo by treatment with dehydroepiandrosterone (DHEA). Similar defects were also observed in human skin as demonstrated by reduced expression of RACK-1 measured by western blot and immunofluorescence. As the DHEA/cortisol imbalance is important during aging, we found that DHEA and cortisol antagonistically act on RACK-1 transcription and translation, and, indirectly, on the LPS-induced cytokine production. *Conclusion:* These observations on the control of a key element in the signal transduction cascade regulating immune function highlight a link with the hormonal changes in cell environment associated with aging, and the complex process of ageing of the skin and its immune system.

Key words: Immunosenescence, aging, cytokines, RACK-1, dehydroepiandrosterone, cortisol, signal transduction.

Introduction

Given the increasing proportion of elderly people worldwide, a better understanding of the causes and mechanisms of immunosenescence is crucial to identify whether prevention might be beneficial to enhance quality of life and to reduce the cost of medical care in old age. The elderly suffer from more frequent and severe community acquired and nosocomial infections than younger people, and they experience poor outcomes from infections in comparison to youngs. Common infections seen in the elderly are infections of skin and soft tissue, urinary tract, respiratory tract, and gastrointestinal tract (1). The increased susceptibility to skin infections is due to age-related anatomical, physiological and environmental factors. The types of organisms that cause skin and soft tissue infections include bacterial, viral and fungal pathogens as well as parasites (2, 3). Cellulitis and infected ulcers are the most commonly encountered cutaneous infections in the elderly. Secondary skin infections are often the result of persistent pruritus associated with increasing dryness of the aging skin. Staphylococcus aureus and beta-haemolytic streptococci are the most common causative organisms of cutaneous infections. By age 70, approximately 70% of persons have at least one underlying skin problems (4). The possibility of the aging organism to respond to external damage and infections is strictly related to the functionality of the immune system, which declines with age.

Immunosenescence

Immunosenescence is due to reduced, deregulated, exhausted immune responses with aging. It is a complex process, not yet fully understood. Both physiological and external factors contribute to immunosenescence. Among the several external factors that can impact immune fucntionality, we can mention life style, malnutrition, smoking, alcholism, comorbidities, etc.. Normal aging is associated with a number of impaired immune responsiveness such as reduction in response to recall antigen, alterations in T cell functions, which contribute to increased vulnerability to infectious disease and

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malignancy, and reduced responses to preventive vaccination in the elderly (5, 6).

The physiological factors contributing to immunosenescence are numerous and complex. They include stem-cell defects, thymus involution, aging of resting immune cells, replicative senescence of clonally expanding cells, defects in antigen-presenting cells, dysfunction in several signal transduction pathways and dysregulation of the cytokine network (6). Our contribution was the observation that both in rodents and humans a key factor in the reduced immune response to external challenge is a defective protein kinase C (PKC) machinery. More specifically, in the absence of a defect in total PKC expression the failing element in the signal transduction cascade is the expression of RACK-1 (Receptor for Activated C Kinase – 1) (7-10). RACK-1 a 36-kDa protein cloned from rat brain, is the best-characterized member of the RACK family. The importance of RACK-1 in PKC signal transduction is related to its function as a scaffold protein. PKC activation involves its translocation from the cytosol to membrane compartments to exert its function (11). RACK-1 modulates PKC activity by stabilizing its active conformation (12, 13). PKCs mediate essential cellular signals required for activation, proliferation, differentiation and survival of immune cells (14). Defective PKC activation with aging has been reported in human monocytes (15, 16), in human T lymphocytes (17, 18), and approximately 50% of elderly subjects have significant reduction in PKC activity in B cells (19).

Within the immune system, RACK-1 also functions as an adaptor recruiting the transcription factor STAT1 to the IFN receptor complex and is a scaffold protein for the IFN-alpha receptor 2/beta-chain of the receptor, Janus kinase 1, and tyrosine kinase 2 (20). RACK-1 may also serve as a scaffold protein in other cytokine systems such as IL-2, IL-4, and erythropoietin as well as the signalling pathways of TNF-R55 (21).

Cytokines are essential for proper antigen presentation, activation of lymphocytes and elimination of invading microorganisms. Alteration of the cytokine network is believed to play a role in the remodeling of the immune system in old age (22). Regarding this aspect, we found that aging was associated both in human and rodent leukocytes with a progressive decline in LPS-induced TNF-α production (7-9). TNF-α is one of the major cytokine produced during an infection, and a decrease in the ability of cells to produce this cytokine is likely to contribute to increased susceptibility to infections. We could demonstrate in leukocytes, by using a specific PKC β inhibitor and RACK-1 antisense oligonucleotides, a key role, although not exclusive, of PKC β signaling pathway in TNF-α production.

A signifcant evidence of the reduced activity of the immune system in the elderly is the defective response to vaccination. Indeed vaccination could protect elderly people against several infections, and the skin is an attractive site for vaccination because it is rich in various antigen-capturing immune cells. Exploration and restoration of age-dependent dysregulation of skin immune responses is, however, necessary for effective vaccination campaigns in the elderly. One of the greatest practical healthcare challenge in the elderly is infact to ensure that vaccinations are otpimally effecitve (23, 24).

Intradermal administration of antigens is a strategy to increase and facilitate their exposure to antigen-presenting cells present in the skin, particularly dendritic cells. Intradermal vaccination, evaluated for influenza, rabies, and hepatitis B, should produce high antibody responses with smaller quantity of antigen. Influenza is a common infection which causes annual epidemics, and in the elderly, is associated with increased morbidity and mortality (25). There is an extensive literature on the effect of aging on response to influenza vaccination in the elderly. It has, however, a moderate impact on influenza-specific immune responses and infection rates in the elderly: while influenza vaccination has more than 80% efficacy in healthy adults, this falls to 17-50% in the elderly (10, 26, 27). Belshe et al found that intradermal injection of a reduced dose of influenza vaccine elicited a signifcant and vigorous response in young and adults, but only weak responses in elderly over the age of 60 years (28). In a study we conducted involving fifty elderly subjects age 63-85 y and fourteen young subjects age 26-41 y, a lower antibody titer to influenza A virus was observed in aged individuals. The sieroconversion factor was 1.08±0.10 in the elderly versus 2.25±0.35 in the young [10]. We also found that the sieroconversion factor was inversely correlates with IL-10 production (linear correlation $r=-0.484$, $p<0.0001$), directly correlated with TNF-α production ($r=0.3997$, $p=0.0012$) and, to a lesser extend, with plasma level of DHEA ($r=0.363$, $p=0.0034$). The decreased TNF-α and increased IL-10 production were both correlated to plasma level of DHEA ($r=0.326$, $p=0.0076$ and $r=-0.490$, $p<0.0001$ for TNF-α and IL-10, respectively). These results suggest that altered cytokine production in elderly subjects at the moment of vaccination can be predictive of a low response to influenza vaccination. Strategies to improve protection afforded by the use of vaccine should include strategies directed to restore altered immune responses associated with aging.

Skin immunosenescence is, therefore, not only relevant for age-associated increase in skin infections and malignancies, but it can also account for defective responses to vaccinations observed in the elderly.

Skin Immunosenescence

One of the major tasks of the epidermis is to defend the organism against exogenous insults. The skin is poised to react to infections and injury, with rapidly acting mechanisms that precede the development of specific immune responses and serve as an immediate defence

system (29). Skin immunosenescence accounts for increased susceptibility and severity in the elderly to cutaneous infections and malignancies, decreased contact hypersensitivity and response to vaccination. Defective cell-mediated immunity, altered number and function of Langerhans cells associated with aging (30, 31) significantly contribute together with cumulative exposure to UV radiation and aging anatomy and physiology to the clinical expression of malignancy (32), increased rate of infections, reduced rate of sensitization and response to vaccination (33, 2).

Although an age-associated decrease in cutaneous immune cell number and function has been reported (33, 34), relatively little is know about the molecular mechanism(s) underlying such defects. We have recently determined that an age-related decline in skin RACK-1 expression was present and it correlated with defective TNF-α production by rat epidermal cells. It is important to mention that TNF-α, together with IL-1, is a key player in the initiation of Langerhans cells (LC) migration (35), and it has been demonstrated that migration of LC is PKC-β dependent (36, 37). If the availability of either TNF-α and IL-1-β, is compromised then LC migration and dendritic cells accumulation in draining lymph nodes and the development of an appropriate immune response is inhibited largely or completely (38). Reduced dendritic cells number and impaired functionality in the elderly represent a hurdle to be overcome in developing successful vaccination strategies.

Skin aging: experimental evidence of reduced RACK-1 expression in human skin with aging

Skin morphological analysis. Aging is associated with increased skin dryness, loss of elasticity and firmness, atrophic changes, smoothness and the appearance of wrinkles. Clinical manifestations of aged skin include xeroses and the occurrence of begnin neoplasms. Consistent with literature data, histological examination of H&E stained abdominal skin sections (Fig. 1A and B) obtained from healthy young (22 y) and postmenopausal woman (60 y) undergoing plastic surgery did not revealed evident morphological alterations in the epidermis of aged skin. Aged and young skins displayed similar keratinocyte stratification. The most obvious changes are at the epidermal-dermal junction, with flattering of the surface contact of the epidermis and dermis resulting from the retraction of the epidermal papillae in aged skin. Collagen and elastic fibers showed marked alterations with age, both fibrous components appear more compact because of a decrease in spaces between the fibers. Collagen bundles appear to unravel, and the individual elastic fibers show signs of elastosis. As described in the literature, other important morphological changes with aging are decrease in number of melanocytes and Langerhans cells (39). On the contrary the counts of T cells in healthy skin from young and old adults are not different (40).

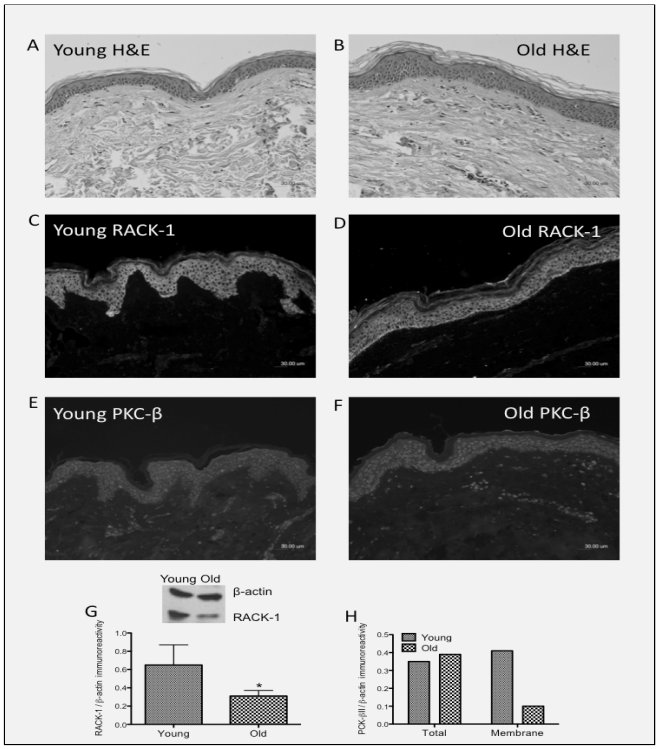

Figure 1. Skin histology, PKC β II and RACK-1 immunofluorescence, and PKC β II and RACK-1 Western blot analysis. (A, B) Haematoxilin and eosin (H&E)-stained skin sections obtained from young (< 50y) and old (> 60 y) females. (C, D) PKC β II immunohistochemistry. (E, F) RACK-1 immunohistochemistry. Photomicrographs are representative of all subjects examined. (G) Western blot analysis of RACK-1 expression and relative densitometric analysis in whole skin homogenate obtained from young and old females. β-actin was used to control for protein loading. Each value represents the mean SD, n=3, Student's t test, with * p< 0.05 vs young. (H) Western blot analysis of total and membrane PKC β II isoform immunoreactivity in whole skin homogenate obtained from young and old females. β-actin was used to control for protein loading. See ref 62 for technical details

Decrease of RACK1 expression with age. To confirm data obtained in experimental animals, a study was designed to determine if an age-related decline in RACK-1 expression was also present in the human skin. As shown in Fig. 1, a decrease in RACK-1 expression was observed both in whole skin homogenate obtained from post menopausal women (> 60 y) in comparison to young whole skin homogenate (< 50 y), as assessed by Western blot analysis and relative densitometric analysis (Fig. 1G), and by immunolocalization of RACK-1 in skin section (Fig. 1C and 1D). Consistent with Western blot analysis, immunolocalization of RACK-1 showed a reduced staining both in the epidermis and dermis of skin obtained from postmenopausal woman if compared to

the skin obtained from young female (Fig. 1B). RACK-1 resulted highly expressed in the epidermis and in skin annexes, while fibroblasts showed a less intense staining, all appeared to be reduced in aged skin.

Consistent with previous results, a similar PKC β immunolocalization in the skin obtained from young and old humans (Fig. 1E and 1F), and PKC β total expression in skin homogenates were observed (Fig.1H). Total expression of PKC β, however, doesn't provide indication of the active state of PKC. PKC activation indeed evokes its translocation from the cytosol to membrane compartments for exerting its function, process mediated by RACKs proteins. As reported in Fig. 1H, despite a similar total expression, a reduced expression of PKC β in the membrane compartments was observed in skin homogenate obtained from post menopausal women ($p<0.05$), which underlying a defective translocation. Importantly, the decreased translocation of the PKC β isoform is consistent with the reduced RACK-1 expression observed, as RACK-1 preferentially interacts with PKC β.

Hormonal balance and signal transduction

Skin aging is affected by growth factor modifications and hormone activity that declines with age (41). The best-known decline is that of sex steroids such estrogen, testosterone, DHEA and its sulfate. DHEA and its sulfate ester DHEA-S are both the secretory products of adrenal glands and the most abundant hormones in the systemic circulation of humans, converted then into androgens and estrogens in the periphery. Several cell types present in the skin are known to be androgen sensitive and express steroidogenic enzymes required to transform DHEA into dihydrotestosterone and 17-estradiol (42). Skin, being a steroidogenic tissue itself, can be affected to a large extent by sex steroids, particularly androgens (43). It has been demonstrated that they can modulate skin thickness, barrier homeostasis, wound healing, sebaceous gland growth and differentiation, hair growth, etc. and it has been proposed DHEA supplementation, both orally and topically, as an effective way to reverse the age-associated deterioration of skin (44-47).

Many studies have now shown that DHEA has significant immune modulatory function, exhibiting both immune stimulatory and anti-glucocorticoid effects (reviewed in 48). The impaired DHEA secretion with aging together with the increase of cortisol results in an enhanced exposure of lymphoid cells to deleterious glucocorticoid actions. DHEA may affect production of Th1 and Th2- associated cytokines, which may be relevant in immunosenesence. Studies in experimental animals and humans have shown that DHEA increases the production of Th1-associated lymphokines, namely interleukin-2 (IL-2) and interferon-gamma by lymphocytes (49, 50). Serum DHEA concentrations in adult male patients with atopic dermatitis had been reported to be significantly lower than those of age-matched healthy male controls (51). Furthermore, preincubation of peripheral blood mononuclear cells with DHEA reduced the IL-4 production induced by concanavalin A (52). The ability to regulate immune function has raised interest in the therapeutic potential of DHEA as a treatment for the immunological abnormalities that arise in subjects with low circulating levels of this hormone, including attempts at reversing the impaired immune response of older individuals to vaccination and restoring immune regulation in patients with chronic autoimmune disease (53).

The impaired DHEA secretion together with the increase of cortisol results in an enhanced exposure of lymphoid cells to deleterious glucocorticoid actions (54). A role of cortisol/DHEA in regulation of RACK-1 expression is presented. The effect of cortisol on RACK-1 expression might contribute to our understanding of the immunosuppresive effects of glucocorticoids. Therefore, an unnecessary or excessive use of glucocorticoids in the elderly may contribute to impaired immune cell activation also through impaired PKC signaling.

We studied the promoter region of the human gene encoding for RACK-1, which is known as GNB2L1, and identified in this region a putative glucocorticoid responsive element (GRE) (55) (see Fig. 2). Experimental evidence suggest that DHEA can upregulate the expression of RACK-1 at transcriptional level (8, 9) while no direct effect can be demonstrated on the promoter of its gene (55). There is, however, evidence that cortisol significantly reduce GNB2L1 promoter activity and reduces RACK-1 mRNA and protein levels (Fig 2). Overall, these data suggest that cortisol at physiological concentrations can inhibit the expression of RACK-1 protein via inhibition of the activity of its gene promoter. Other experiments (56) demonstrate that preincubation of THP-1 cells with DHEA counteract the effect of cortisol. The data are consistent with an effect that does not involve direct pharmacological antagonism of DHEA vs cortisol, rather a mechanism requiring long term adaptation of glucocorticoid receptor activity involving slow nuclear translocation with loss of transcriptional activity. DHEA therefore reduces the effect of cortisol on GNB2L1 and consequently on RACK-1 mRNA and protein. Furthermore the levels of TNF-α released in response to LPS challenge can be used as a measure of PKC/RACK-1 dependent activation of immune functions. While cortisol induces a reduction of response in term of TNF-α release, pretreatment with DHEA was able to prevent cortisol inhibitory effect, restoring TNF-α release to control values. It is conceivable then that changes in the balance of these two hormones during aging (10) reflects itself on the expression and regulation of RACK-1 and its correlated immune functions.

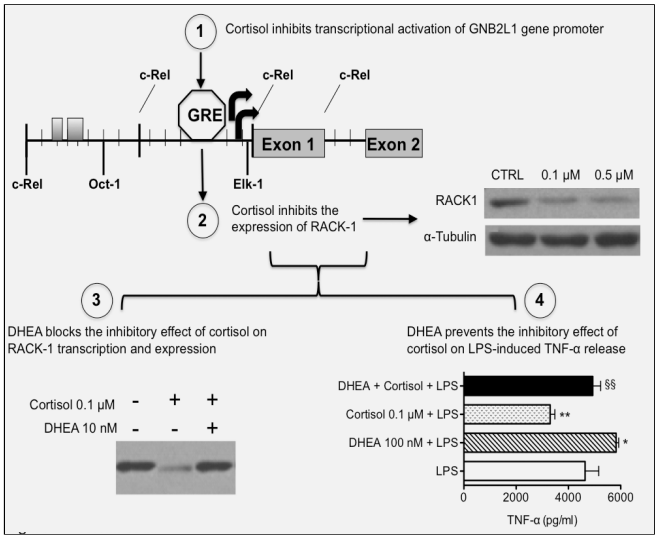

Figure 2. DHEA contrasts the effects of cortisol on RACK-1 expression and LPS-induced TNF-α release in THP-1 cells. A glucocorticoid responsive element (GRE) on the promoter of human RACK-1 (1) responds to cortisol by inhibiting RACK-1 transcription and expression (2). DHEA administration restores RACK-1 level and immune functions, indicating that this hormone behaves as a positive RACK-1 regulator (3, 4). DHEA, endowed of antiglucocorticoid properties, can inhibit the negative regulation of RACK-1 expression induced by cortisol via an indirect action probably by interfering with glucocorticoid receptor interaction with GRE sequence, restoring RACK-1 to control level (3) and preventing the inhibitory effect of cortisol on LPS-induced TNF-α release (4). Each value represents the meanSD, n=3, Dunnett multiple comparison post test with *p<0.05, ** p<0.01 vs LPS treated cells and §§p<0.01 vs cortisol treated cells. See ref 55 for technical details

The relevance of DHEA in the control of RACK-1 expression is also confirmed by its ability to induce RACK-1 expression in human and animal skin cells as shown in Table 1. As previously demonstrated both in human and rodent immune cells (7-9), in both primary human fibroblasts as well as in the human keratinocyte cell line NCTC 2544, the treatment with DHEA induced a dose-related increase in RACK-1 expression as assessed by Western blot analysis. The effect of DHEA in the human promyelocytic cell line THP-1 is shown to confirm its immunostimolatory effect. Importantly, similar results were also obtained treating primary rat epidermal cells obtained from young (3 m) and old animals (> 18 m) with DHEA 100 nM, confirming the ability of epidermal cell types, including cells obtained from old animals, to responde to DHEA increasing RACK-1 expression. This may support from a mechanistic point of view some of beneficial effects of DHEA supplementation in aged skin.

Interestingly, it has been recently demonstrated that PKC-β acts as an intracellular receptor for DHEA-S in human neutrophils, which has important implications for immunosenescence, as it is associated with reduced neutrophil superoxide generation in response to pathogens (57). This evidence further supports the important of PKC signaling in aging.

Table 1
Effect of DHEA on RACK-1 expression in different cell models

DHEA (nM)	THP-1	NCTC	Human fibroblasts	3 m old rat epidermal cells	18 m old rat epidermal cells
1	12322	9127	9315	n.d.	n.d.
10	1685**	8814	9517	n.d.	n.d.
100	19131**	13014*	1292*	13926	12815
1000	1535*	13916**	1275*	n.d.	n.d.

Cells were treated with DHEA for 24 h. RACK-1 immunoreactivity was evaluated by Western blot analysis. Results are expressed as RACK-1/β-actin (% of control). Each value represents the mean±SD, n= 4 independent experiments or animals in the case of rat epidermal cells. *p<0.05, **p<0.01 vs control. The level of expression of RACK-1/β-actin in control cells is 0.580.10 in NCTC cells, 0.300.06 in THP-1 cells, 0.480.05 in human fibroblasts, 0.600.19 in 3 m old rats, and 0.390.14 in 18 m old rats. n.d. = not done. See ref 62 for technical details.

Conclusions

A speculation, based on analogies with leukocytes, can be made that hormonal changes in the cell environment associated with aging (i.e., the age–related decline in the levels of dehydroepiandrosterone) are likely to contribute to the loss of RACK-1 expression (8, 9). The reduction with aging in RACK-1 expression was observed in all leukocyte subpopulations (7-9), brain (58-60), and, as reported here, in skin cells, indicating that it may reflect a defect common to many cells types. Certainly, the defect cannot be ascribed to changes in the epidermal structure and differentiation as shown by histological analysis. Further studies are necessary to understand the role and meaning of the changes in RACK-1 expression with aging in dermis and skin annex.

Overall, this paper contributes to the understanding of the complex process of aging of the skin and its immune system and identifies a decline in RACK-1 expression with aging. Age-related alteration in PKC signaling and TNF-α release provide a new understanding of the consequences of aging on skin immunology. The identification of changes in the skin immune system will allow the elaboration of better strategies for the prevention of infective diseases and response to vaccination in the elderly (61). Even in the presence of intrinsic defects in cells involved in the immune response, it is possible that these may be overcome by measures that are relatively easy in principle (i.e. to test whether hormonal treatment can restore immune functions declined with aging), yet these principles need to be clearly tested in appropriate clinical protocols and settings.

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