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PROSPECTS FOR CUTANEOUS WOUND HEALING IN AGEING SKIN.

A WORKING HYPOTESIS: CHITOSAN AND CERAMIDES

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Synopsis

In elderly people the cutis is characterised by structural fragility due to causes ranging from the thinning of the lipid-epithelial stratum to reduced dermal vascularisation, in particular, reduction in the content of all lipids - due mainly to sphingolipids and ceramides, especially at the level of the stratum corneum - and in the extracellular matrix because of greater degradation and reduced synthesis.
We set out to investigate the functional implications of a molecular association of lipopolysaccarides applied to elderly people wound healing with a veiw to re-estabilishing the cutaneous microenvironment which in these subjects is unbalanced and at risk. Water-soluble chitosans in the class of N-carboxyalkyl chitosans possess antimicrobial activity including candidacidal activity, and regenerated chitins and water-soluble chitosans are being used as wound dressings which facilitate the formation of new, ordered tissue. Based on these observations, the combined action of ceramides and chitosan appears to meet the requirements of the elderly, and may constitute *in vivo* a natural association capable of improving the cutaneous microenvironment.

Riassunto

Negli anziani la cute si caratterizza per una fragilità strutturale principalmente dovuta ad un assottigliamento dell'epitelio, del film lipidico di superficie e ad una riduzione della vascolarizzazione.
In particolare la riduzione del contenuto di lipidi a livello dello strato corneo e della matrice extracellulare si deve ad una maggiore degradazione e ad una ridotta sintesi principalmente di sfingolipidi e ceramidi.
È nostra intenzione condurre uno studio per valutare le implicazioni funzionali che una nuova associazione di lipopolisaccaridi potrebbe avere nella cicatrizzazione di soggetti anziani al fine di ristabilire il microambiente cutaneo che in essi è alterato e a rischio. I Chitosani solubili della classe degli N-carbossialchilchitosani, che possiedono attività antimicrobiche e anti-candidosiche, sono già stati utilizzati come presidi per la cicatrizzazione favorendo una ordinata riparazione dei tessuti.
L'utilizzo di un presidio costituito da ceramidi e chitosani sembra rispondere alle esigenze della cute dell'anziano costituendo un'associazione probabilmente in grado di migliorare il microambiente cutaneo.

As major costituents of intercellular lipids, sphingolipids are important determinants of stratum-corneum water holding and permeability barrier function (1). The epidermal permeability barrier is therefore provided by intracellular lipids forming multiple membrane bilayers in the stratum comeum. An alteration in the lipid balance should thus be considered a predisposing factor for dry skin, which in the elderly is often the cause of itching sine materia (2).

In elderly people the cutis is characterised by structural fragility due to causes ranging from the thinning of the lipid-epithelial stratum to reduced dermal vascularisation, in particular, reduction in the content of all lipids - though mainly of sphingolipids and ceramides, especially at the level of the stratum corneum - and in the extracellular matrix because of greater degradation and reduced synthesis (2).

In this medico-cosmetological context, we set out to investigate the functional implications of a molecular association of lipo-polysaccharides applied to the cutis of elderly people with a view to re-establishing the cutaneous microenvironment which in these subjects is unbalanced and at risk. Previous experience on tissue repair features in elderly subjects indicate that their "thin skin", especially in women with (post-menopausal) estrogen deficit, while showing slow epithelial and fibroblastic proliferation, usually allows the stromal matrix to be repaired without antiesthetic scars.

In these subjects precarious skin hydration constitutes both cause and effect of tissue dystrophisms which depend on its reduced reactivity to damage and on the gradual thinning of the hydrolipidic stratum (2).

Based on these observations, the combined action of ceramides and chitosan could appears to meet the requirements of the elderly, and might constitute *in vivo* a natural association capable of improving the cutaneous microenvironment of these and other subjects (!).

Interestingly, sphingolipid metabolities participate in key events of signal transduction and cell regulation. In the sphingomyelin cycle, a number of extracellular agents and insults (such as tumour necrosis factor, fas-ligands, and chemotherapeutic agents) cause the activation of sphingomyelinases, which act on membrane sphingomyelin and release ceramide (3, 4).

In the first place, membrane glyco and sphingolipids are important in the modulation of activity, adhesion to the substrate and cell morphology; indeed, the morphological differentiation of the epidermis rests on a high ceramide content (5). We thus set out to test a cosmetological combination of ceramides, which act prevalently at epidermal level, with chitosans. As regards the latter, similarities between some modified chitins and hyaluronic acid have been reported and the morphogenetic role of glycosaminoglycans is known to lead to cellular replication, stromal collagen network and biofunctional characteristics close to normal.

Chitosans are polysaccharides derived from chitin, which as raw material is amply available and comes from a number of crustaceans. It can be described as a copolymer of N-acetylglucosamine and D-glucosamine. Medical-grade chitins and chitosans are commercially available. To enhance their solubility, they can be chemically or enzymatically modified based on mild treatments in aqueous media, leading to highly purified products. This is particularly true of the modified chitosans in the class of N-carboxyalkyl chitosans (7).

Further properties of these molecules are: a) high capacity to adsorb water, the modified chitosans being able to modify the water structure, b) absence of noxious effects on healthy tissues, and c) reparative action on wounded tissues.

Water-soluble chitosans in the class of N-carboxyalkyl chitosans possess antimicrobial activity including candidacidal activity, and regenerated chitins and water-soluble chitosans are being used as wound dressing which facilitate the formation of new, ordered tissue. Furthermore chitosans are chelating agents and prevent the adsorption of toxic heavy metal ions accidentally present in solutions (7).

In laboratory animals, N-carboxybutyl chitosan, a water-soluble chitin derivative, has been demonstrated to induce the formation of ordered repair tissue where collagen bundles had a regular direction; wounds treated with N-carboxybutyl chitosan did not show evident

scar formation or wound contraction.
The results of some of our studies - confirming the data discussed above - are reported below (6).

PATIENTS, CLINICAL ASPECTS AND MORPHOLOGICAL INVESTIGATIONS

Four plastic-surgery patients, two males and two females, aged 30 to 50, underwent medium-thickness dermo-epidermal square explants (50 cm^2) performed with the aid of a dermotome on the frontal part of the thigh. This donor site was treated with N-carboxybutyl chitosan (area A). A similar explant was made close to it (control site, area B) and treated with phytostimuline gauze. Both sites were medicated after 7 days. On days 10 and 30, when the surgical wounds were clinically healed, biopsies were obtained from both areas.

N-carboxybutyl chitosan

Medical-grade chitosan was obtained from Alaska king crab chitin and was further modified into water-soluble N-carboxybutyl chitosan according to our own procedure (6). The resulting solution was dialysed and freeze-dried to produce soft and flexible pads which were sterilised and applied to the wound. Analytical data were: M_w 720,000, determined by laser light-scattering spectrophotometry; degree of N-carboxybutylation 0.27, determined by high-pressure liquid chromatography; ashes at 600°C, <0.1%, by gravimetry, and pH of the 1% solution 6.2.

Transmission electron microscopy investigations

Specimens were fixed in 2.0% glutaraldehyde in 0.15 cacodylate buffer, post-fixed in 1% OSO_4 in cacodylate buffer and dehydrated in increasing concentrations of ethanol. The samples were embedded in Araldite. Semithin and ultrathin sections were cut using a Reichert Ultracut E microtome; ultrathin sections were counterstained with uranyl acetate and lead citrate and observed with a Zeiss EM 109 electron microscope.

Clinical aspects

One of the advantages of using N-carboxybutyl chitosan in wound management is that it gelifies in contact with the wound fluids, forming a layer which provides an outstanding protection of the newly-formed tissues against mechanical damage. The outer surface of the pad acquires the aspect of a crust and provides protection against secondary infections by virtue of the bactericidal action of the polymer. Infections were not observed in any of these fours patients.
During the healing period, the square shape of the wound was preserved, though its size decreased progressively, while in control wounds the square shape was soon lost after traditional medication. Complete healing occurred after 8 days for area A and 7 days for the control area in all patients.

Morphological investigations

- **Repair process in control areas.**
  Ultrastructural analysis evidenced the presence of repair tissue showing a clear mesenchymal cell component represented by polygonal elements (i.e. fibroblasts) and a small number of inflammatory cells. The cell distribution was irregular and the relationship between fibroblasts and collagen network did not provide evidence for a preferential histoarchitectural pattern. Vascular structures were generally numerous and arranged in layers. The presence of some layers of granular cells was also observed, as was the presence of partly or fully keratinized layered elements. These features were the expression of a completed epidermal maturation process.
- **Repair process in the presence of N-carboxybutyl chitosan.**
  Ultrastructural analysis identified fibroblasts of elongated shape arranged according to precisely oriented, parallel lines. Vascular structures were

largely present while the inflammatory cellular component was occasional. The collagen network, though rather loose, showed a regular distribution. The general aspect of the derma gave an overall impression of histoarchitectural order which was more evident than in controls. The epidermis exhibited in general a linear junction with the derma, without marked offshots. Overall, the epithelium was organised and cytologically normal, even though the malpighian layer appeared less extended than in controls, as did the whole multilayer of the epidermis. The skin reached its final differentiation with superficial features of keratosis with both types of dressing. After 1 month, more features of the maturation process were identified at morphological analysis (Fig 1).

Prospects

The early steps of skin-tissue repair are sustained by fibroblast proliferation, collagen deposition, angiogenesis and subsequent epithelisation.
In this phase, N-carboxybutyl chitosan gel favours the formation of a loose connective tissue rather than large and dense fibre bundles, facilitating the diffusion of factors and substances and cell proliferation. Then, in the later stages of the normal process of wound healing, when collagen synthesis declines and high oxygen tension is no longer required, many new vascular channels regress; the wound becomes usually avascular and undergoes a transformation into a scar with impaired tissue elasticity. Regression of angiogenesis takes place as soon as chitosan is no longer administred or has been adsorbed; nevertheless, the resulting connective tissue is regularly and orderly structured, without noticeable scars and is endowed with good functionality, i.e. tensile strength. In fact, one of the functions exerted by N-carboxybutyl chi-

Fig. 1. Regenereted skin by chitosan gel: note the evident keratinocytes differentiation even if lipid barrier reconstitution is poor (TEM, 20000x).

tosan appears to be the limitation of the process of wound contraction due to the production of a loose collagen network and the inhibition of large-bundle formation, a point of similarity with heparin and the ability to keep high hydration conditions, a point of similarity with glycosaminoglycans (6, 7).
As far as re-epithelisation is concerned, the fibrin clot acts as a scaffold for migrating epithelial cells. Epithelial cell migration takes place through the fibrous stromal proteins, and epithelial cells secrete collagenase to pass through that stromal medium. For wound healing to take place, a tridimensional supporting lattice is very important; N-carboxybutyl chitosan has been reported to favour rapid re-epithelialisation.
As regards ceramides, several experimental approaches suggest an important role for ceramides in regulating such diverse biological responses as cell-cycle arrest, apoptosis, and cell senescence. *In vitro*, ceramide activates a serine threonine protein phosphatase, and in cells it regulates protein phosphorylation as well as multiple downstream targets (such as interleukin-converting enzyme (ICE)-like proteases, stress-activated protein kinases, and the retinoblastoma gene product) that mediate its distinct cellular effects. This spectrum of inducers of ceramide accumulation and the nature of ceramide-mediated responses suggest that ceramide is a key component of intracellular stress-response pathways (8).
The assessment of barrier function in aged epidermis under basal conditions is known to be misleading, since both barrier integrity and barrier repair are markedly abnormal. As mentioned above, these functional changes can be attributed to a global deficiency in stratum-corneum lipids, resulting in thinning lamellar bilayers in stratum-corneum interstices. The relationship between higher ceramide skin content and improvement in skin hydratation evidences the important role of ceramides in the maintenance of a physiological barrier function of the skin by controlling its hydratation status and counteracting stressing stimuli (9). With a view to attaining optimum cutaneous repair, also the superficial lipid layer should thus be completely reconstituted, especially in elderly subjects where a decrease in stratum-corneum lipids is a major etiological factor for atopic dry skin and a primary event in the transformation into aged dry skin (10). We believe that this goal will be achieved by combining chitosans and ceramides, even though the experience of other researchers indicates that a series of problems - connected mainly with the adsorption of ceramides - remain unresolved.

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