Rhamnose-rich and fucose-rich oligo- and polysaccharides (RROP-s and FROPs), agonists and antagonists of cell-membrane receptors as new active principles against skin aging.Robert L.[1], Robert A.M.[1], Gesztesi J-L.[2], Luppi E.[2]

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Summary

Rhamnose-rich (RROP-s) and fucose-rich (FROP-s) oligo- and polysaccharides were prepared and extensively characterised by physical and chemical procedures [1,2] and compared to L-fucose. Their biological properties were then studied on human skin fibroblast cell cultures, human skin explant cultures and on hairless rat skin, using a variety of cell-biological, biochemical and computerised morphometrical procedures. Among the most important properties we could establish, the following are of particular interest for the tretment and prevention of age-dependent modifications of human skin (loss of skin-tissue, cells and matrix, wrinkle formation and others) : stimulation of cell proliferation (by ^{3}[H]-thymidine incorporation and the MTT test), scavenging of reactive oxygen species (ROS) using several different procedures, and protease (MMP-2 and MMP-9) down-regulation. A topical preparation, using RROP-s and FROP-s, and/or L-fucose, was shown to increase cell proliferation, dermal matrix synthesis, efficient scavenging of ROS-s and to increase also the thickness of dermal tissue when applied for 4 weeks on hairless rat skin, accompanied by the densification of collagen bundles as well as by an increase of elastin synthesis. Using fluorescent labeled FROPs, it could be shown that these oligosaccharides react with cell-membrane receptors and especially with the elastin-laminin-receptor and the fucose-mannose receptor, but they penetrate also in the cell nucleus, suggesting the possibility of a direct action on the regulation of gene expression. When applied to the human skin of a team of voluntary women encompassing all age-groups, the efficiency of FROP-containing preparation could be confirmed using indentometry and computerised evaluation of skin micro-relief, as well as evaluation of periorbital wrinkles. It appears therefore that these preparations correspond to all the requirements of active anti-aging principles.

I. Introduction

Skin aging became an important issue of our rapidly aging society. 20 to 35% of the general population in all advanced and in most advancing countries is above 65 years. The most rapidly increasing fraction of this population are the very old (centenarians [3]). This fact creats an important market for anti-aging skin products adressing an increasingly exigent population. Modern research carried out in some laboratories is taking up this challange with increasing efficiency. Before describing the results obtained in our laboratory along these lines of investigation, we have to discuss in some detail the basic cellular and molecular mechanisms involved in skin aging.

Cellular and molecular mechanisms involved in skin aging.

In order to define the most significant tests for screening studies, the first important issue is to define the mechanisms which underly skin aging. Aging of tissues is a complex process which involves the aging of cells, of extracellular matrix (ECM) and of cell-matrix interactions [4,5]. These interactions are mediated by receptors as the integrins or the elastin-laminine-receptor (ELR [6]). Both types of receptors (as well as some others) are involved in the aging process. Cell-aging, as studied in cell cultures, follows the principles elaborated by L. Hayflick [7] and is explained essentially by the telomer-telomerase-centered mechanisms [8,9]. Cell aging affects cell divisions both for keratinocytes and fibroblasts which is progressively slowing down with age. As shown however by the slow but efficient wound healing of elderly subjects, it remains sufficient to close surgical or accidental wounds.

An other important aspect of cell aging is the progressive modification of the « program » of the biosynthesis of ECM components. This is an important aspect of skin aging because of the rich ECM of dermis. Loss of dermal tissue measured as the skin thickness of biopsy specimens from sun-protected sites gave an average estimate of 7% of the original skin thickness (extrapolated to birth) lost every 10 years [10]. This leaves less than the one third of the original skin thickness at about 90 – 100 years (Fig.1a.). This loss of skin tissue is the combined result of cell loss and mainly of

reduced cell-biosynthetic activity, as well as of increased proteolytic activity. Age-dependent loss of collagen (Fig1.b.) is the result of the combined effect of decreased biosynthesis and increased degradation, as the result of the age-dependent increase of the local production of matrix degrading enzymes. As shown on Fig.2., elastase-type endopeptidase activity is steadily increasing with chronological age and also during in vitro aging, with increasing cell passages as shown with human skin fibroblasts [11,12]. Decreasing matrix biosynthetic activity combined with increasing matrix degradation are the two essential ingredients of skin aging. Besides proteolytic enzymes, reactive oxygen species comprising free radicals as hydroxyl radical, superoxide and hydrogen peroxide represent another important source of skin degrading agents. ROS-production is both an intrinsic, cell-dependent process and also a photochemically, UV-induced mechanism. It was shown however, that UVA-induced free radical production was much more important than UVB-induced production [13], is maximal at the skin surface and decreases rapidly towards the dermis. The metabolic generation of ROS is however cell-dependent, essentially of mitochondrial origin, and was shown to increase with age, together with a decrease of the cellular scavenging activity [14,15]. We could show that hyaluronan, one of the most important glycosaminoglycan components of skin, is highly sensitive to free radical degradation. This reaction could be used for the quantitative determination of free radical generation [16,17]. Hyaluronan is produced both in the dermis and epidermis and is involved in a number of important biological properties of skin tissue, such as hydration, control of molecular traffic, activation of MMP-2 and MMP-9 and others [18,19].

Bedsides these mechanisms concerning matrix production and matrix degradation, there is another important aspect of skin homeostasis, the fine adjustment of the relative rates of the expression of genes coding for the ECM-components, collagens, elastin and others [20]. In this respect receptor-mediated cell-matrix interactions play a crucial role. This receptor-mediated information exchange between the cells and the surrounding ECM-components is progressively deteriorating with age, as we could demonstrate in our experiments with the elastin-laminin-receptor [21,22,23]. In cells from old individuals (>65 years) this receptor appeared to be uncoupled from its normal transmission pathway as established on circulating white blood cells and endothelial

cells or fibroblasts. This transmission pathway is shown schematically on Fig.3. One of the most conspicuous results of this uncoupling of ELR, shown on Fig.4., is the loss of its coupling to the G_i –component of its transmission pathway, accompanied however by an increased free radical production. This can easily damage the cell-membrane and account for the loss of the calcium homeostatic regulations of the cells, demonstrated experimentally on PMN-leucocytes obtained from aged-pathological donors [24]. The above summarised mechanisms, cell proliferation, matrix production and degradation, ROS-scavenging are the reactions we explored systematically for the characterisation of new active principles.

III. What is meant by « anti-aging » priciple ?

This is a somewhat abusive (but largely used) term. It would be more appropriate to speak about the slowing down of aging processes. The demonstration of « peace-meal » aging of tissue functions (« vieillissement en pièces détachées » in French [25]) shows clearly that different tissues and functions age at different rates. Some functions decrease rapidly with age, others much more slowly. Articular cartilage looses rapidly its biomechanical characteristics, most elastic functions as accomodation of the eye lens, elasticity of blood vessels , of the lung or of the skin decline also relatively rapidly. Other functions, related essentially to the central nervous system, decline more slowly. This is true also for the skin, some of its components decrease rapidly (skin collagen and glycosaminoglycans), others may even increase with age, as fibronectine [26,27]. The result is a progressively changing macromolecular composition of skin matrix as demonstrated also by the age-dependent modifications of its rheological properties [28,29].

All the above described factors were taken in consideration for the elaboration of some new active principles designed to counteract the above described mechanisms underlying skin aging. In the present experiments we tested rhamnose- and fucose-rich oligo- and polysaccharides (RROPs and FROPs). The biological origin and chemical preparation and characterisation of these substances was recently described [1,2]. Here

we shall concentrate on the biological-biochemical characteristics of these substances in relation to the above described aging mechanisms.

IV. LABORATORY STUDIES IN VITRO, EX VIVO AND IN VIVO

IV.1. Effect on free radical mediated degradations. RROPs and FROPs as efficient free radical scavengers

For these experiments we used the free radical mediated degradation of hyaluronan, a previously described procedure [16,17]. The viscosity of a 1 mg/ml hyaluronan solution was recorded as function of time in a cylindrical rotating viscometer (Fig.5.). When a highly diluted solution of Ascorbate-$FeCl_2$-EDTA (the Udenfriend reagent) was added, a rapid fall of viscosity could be demonstrated. This reagent releases $OH^{\bullet}$ radicals and produces a rapid cleavage of the hyaluronan chains as shown by the rapid drop of its viscosity. In the presence of low concentrations of RROP this drop of viscosity is slowed down, with a plateau at increasing RROP concentrations. This shows a very efficient free radical scavenging activity of RROPs (Fig.6.).

When similar experiments are carried out with FROPs, the scavenging effect is smaller at the lowest concentrations, but there is a steady increase with increasing FROP concentrations (Fig.6.).

These results substantiate a good complementarity of these two compounds as free radical scavengers, RROPs active already at very low concentrations, but with a rapid saturation, FROPs less active at low concentrations, but increasing their efficiency at higher concentrations.

Using an other, independent test, we could confirm the efficiency of FROPs as protectors against ROS-mediated cell-death. We could show that ascorbate at millimolar concentrations is cytotoxic [30]. This effect is the result of the dose-dependent inhibition by ascorbate of fibronectin biosynthesis, mediated by ROS as shown by the

protection in presence of catalase and SOD. FROPs were shown to protect efficiently human skin fibroblasts against the ascorbate mediated cell-death [31].

Taken all together, these results show, that RROP and FROP are efficient scavengers of free radicals.

IV.2. Effect on cell proliferation

For these experiments human skin fibroblasts were used and cultured as described [1]. The effect of several FROP-preparations (for details see ref.[1]) was compared to that of the original high molecular weight polysaccharides (Fucogel®) as well as to that of L-fucose. The original polysaccharide produced a strong stimulation of proliferation as shown on Fig.7. At a 10 μg/ml concentration this polysaccharide gave a more than 100% increase in proliferation as compared to control cultures, using ^{3}H-thymidine incorporation ($p<0,001$). At higher concentrations (100 μg/ml) less stimulation was obtained (+61%). The effect of L-fucose was more modest but significant, at 1 μg/ml +19% and at 10 μg/ml +35%. FROP-3, used currently in topical preparations, gave at 1 μg/ml +48% stimulation and at10 μg/ml +31%. Both for the high molecular weight polysaccharide and for the oligosaccharides the lower concentrations gave the most important stimulation.

IV.3. Effect on skin matrix biosynthesis

As mentioned in the introduction, aging is accompanied by a progressive loss of skin extracellular matrix, as exemplified by the decrease of skin thickness (Fig.1.). Stimulation of matrix biosynthesis is therefore an important quality of active anti-aging ingredients. We shall describe the effects of the two substances tested, RROP and FROP on extracellular matrix biosynthesis

IV.3.1. Effects in vitro

a) protein and collagen biosynthesis

We tested the biosynthesis of two major matrix components, proteins and glycosaminoglycans. Protein biosynthesis comprises also collagen neosynthesis, tested by the incorporation of radioactive proline in macromolecular proline (total proteins) and macromolecular hydroxyproline (collagens). Glycosaminoglycan biosynthesis was estimated by the incorporation of radioactive glucosamine in glycosaminoglycans, identified by their specific degradation with selective endoglycosydases [32]. We could show that RROP strongly increased ^{3}H-proline incorporation in total neosynthesised proteins by human skin fibroblasts in culture (Fig.8.). From this total incorporation about 3,5 % concerned collagens. In presence of 1μg/ml RROP ^{3}H-proline incorporation increased by a factor above 500x in total proteins, and by a factor of 13x in collagens. At 10 μg/ml RROP produced a further increase of protein and collagen biosynthesis, to a level of about the double of the stimulation by 1 μg/ml.

FROPs also proved to be efficient in this respect. As in many topical preparations this polysaccharide is combined with vitamin C, retinol and vitamin E, their combined effect on protein and collagen biosynthesis was therefore also investigated. We tested separately protein and collagen biosynthesis by ^{3}H-proline incorporation, as well as collagen accumulation in cell cultures by a specific colorimetric procedure [33]. Both methods gave comparable results. Curiously L-fucose alone gave a slight but significant inhibition of collagen biosynthesis and accumulation. This was not the case for the FROP preparation, it potentiated the action of ascorbate on collagen biosynthesis. It also counterbalanced, corrected the retinol-induced inhibition of collagen biosynthesis (Fig.9.). As can be seen on Fig.9., at 10 μg/ml FROP-3 increased significantly ^{3}H-proline incorporation in cell-associated collagens and non collagen proteins. A much more modest effect was seen with L-fucose. As shown on Fig.10., retinol increased incorporation of ^{3}H-proline both in collagen and in non collagen proteins excreted by fibroblasts in the culture medium. This increase was much more important in presence of 10 μg/ml FROP-3. L-fucose did not potentiate the effect of retinol.

b) Glycosaminoglycan biosynthesis

We used ^{3}H-glucosamine incorporation followed by the selective degradation of individual GAG-s as described [32]. Both L-fucose and FROP-3 stimulated incorporation in some selective GAG-s, this effect was however strongly age- (passage number-) dependent. L-fucose stimulated incorporation in heparan sulfates at the 9th passage of fibroblasts by 20%. FROP-3 stimulated incorporation in dermatan sulfate in a dose-dependent fashion: +67% at 1 μg/ml and +128% at 10 μg/ml [34]. Hyaluronan biosynthesis was also stimulated by about 27% [34].

These results taken together strongly support our claim that the rhamnose-rich and fucose-rich preparations stimulate the biosynthesis of several macromolecular components of skin extracellular matrix.

IV.3.2. Effects in vivo

In order to appreciate the effect of these two compounds on in vivo matrix biosynthesis, we determined their effect on the thickness of hairless rat skin after four weeks of local application. This effect was compared to that produced by L-fucose. After 4 weeks of local application we could demonstrate by computerised image-analytical methods on skin biopsies a significant increase of skin thickness, both for the epidermis and for the dermis (Fig.11.). This increase is the combined result on cell proliferation and on matrix biosynthesis.

Another important aspect of the in vivo effect of L-fucose concerned the relative density of collagen fiber-bundles in the dermis. This was also evaluated by image-analytical procedures. As shown on Fig.12. local treatment with L-fucose considerably increased the density of the dermal collagen fibers as shown by the left-shift of the histogram of the fiber density distribution profile.

These results confirm those obtained with in vitro methods and show that L-fucose and the two oligo- polysaccharide preparations strongly stimulate in vivo also the biosynthesis of dermal matrix, as well as the proliferation of the cellular components of skin.

IV.3.3. Effect on elastin biosynthesis

Elastic fibers represent an important component of skin matrix. We could show that the vertical fibers of young skin are progressively degraded and replaced by mostly horizontally running thicker elastic fibers. Although total elastin fiber surface density of human skin is increasing with age, its elasticity is decreasing [35]. This is due to qualitative modifications of aging elastic fibers, which get progressively saturated with Ca-salts and with lipids.

We therefore tested the effect of L-fucose and of FROP-3 on elastin fiber formation in rat skin and on tropoelastin biosynthesis by fibroblasts in culture. Both tests gave positive results. As shown on Fig.13., tropoelastin biosynthesis was stimulated by L-fucose and even more by FROP-3. The morphometric evaluation of elastic fiber surface density on histological sections from topically treated rat skin confirmed the stimulation of skin elastin fiber deposition in the treated skin.

V. STUDIES ON HUMAN VOLUNTARIES

In further experiments the above described biological properties of the polysaccharides were tested and extended to the skin of human voluntaries.

V.1. Effect on human skin.

A cosmetic preparation containing as active principle FROP-3 (and more recently also RROP) was tested on 20 voluntary women representing a wide age-spectrum with different skin
qualities. The following tests were performed:

a) indentometric evaluation of skin firmness (hydration) and elasticity, as described previously [37]
b) skin surface relief by computerised image analytical procedures according to a method worked out in our laboratory [38]
c) evolution of periorbital wrinkles (crow-foot) by measurement of length-width relations on replicas using an image-analytical procedure [39]

These tests were carried out before and after four weeks of application (twice daily, with suppression of all other local treatments), accompanied by the measure of skin surface pH and by sebumetry. All these tests showed a considerable amelioration of these skin rheological parameters, as shown on Fig.14.

V.2. Lasting effects

A further test concerned the permanence of these improvements. Five of the twenty women accepted to abstain from any further skin treatment for two more weeks, and their above mentioned skin parameters were evaluated one and two weeks after the end of the four weeks test period. It appeared that four out of the five women kept an improved skin firmness (hydration) and elasticity. The only woman who did not, was a heavy smoker. Skin elasticity decreased slowly during those two weeks after the last application of the FROP-containing preparation, but remained however still well above those measured before the application of the FROP-containing preparation (Table I.).

Similar favourable results were obtained with the two other tests mentioned, skin microrelief and periorbital wrinkles. After four weeks of treatment with the FROP-containing preparation the number of polygons per unit surface increased, suggesting an efficient reversal of the age-dependent loss of skin surface relief. This depends largely on the structure and composition of the underlying skin tissue and does reflect its improvement. The average improvement of the skin surface relief was of the order of 50%.

V.3. Periorbital wrinkles

Similar favourable results were obtained on the evolution of periorbital wrinkles. The following parameters were recorded on replicas:

1) the number of deep wrinkles
2) the length of each wrinkle
3) average width of each wrinkle
4) total cumulative length of all wrinkles
5) average width calculated for all wrinkles (expressed in units of 0,1 mm)
6) an index obtained by multiplying total length with average width for all wrinkles.

As shown on Fig.15., it appeared that for most women (>65%) the above mentioned wrinkle parameters changed favourably as a result of the treatment with the FROP-containing preparation. The details of all these investigations on human voluntaries will be described in future publications (in preparation).

VI. Discussion

The above described and succently summarised favourable results obtained with the oligo- polysaccharide preparations, designated RROP-s and FROP-s, need to be explained in terms of mechanisms of action at the level of the cellular and molecular components of the skin. We could show with fluorescent-labeled FROP-preparations, that they have two major sites of interaction with human skin fibroblasts (Fig.16.): the cell membrane and the nucleus. Interaction with the cell membrane is maintained even for formol-fixed cells, but nuclear penetration is suppressed. Interaction of FROP-s with cell membrane components appears to concern two types of receptors: the elastin-laminin receptor and the fucose-mannose receptor (1). The presence of a specific α-L-rhamnose recognising receptor was demonstrated on keratinocytes [40]. Detailed study on the transmission pathway of these receptors suggested a plausible explanation for the action of FROP-s and RROP-s at the level of the message-transmission between skin cells and extracellular messages and also at the level of cell-matrix interactions [1]. The

nuclear penetration of FROP-s, about 8-times more intense (as estimated by the measurement of fluorescence intensity) suggests a direct action on gene-expression and regulation. Further studies are indicated in order to fully elucidate the mechanisms of the above summarised remarkable "anti-aging" properties of RROP-s and FROP-s.

References

1. Péterszegi G, Isnard N, Robert AM, Robert L,
Studies on skin aging. Preparation and properties of fucose-rich oligo- and polysaccharides. Effect on fibroblast proliferation and survival.
Biomedicine & Pharmacotherapy **N°Vol** (2003) in print

2. Rhamnosoft® Polysaccharide Attenuateur de l'Inflammation
(2001) Solabia, France, Bulletin technique

3. Allard M, Robine J-M,
Les centenaires français. Etude de la Fondation IPSEN, 1990 – 2000,
(2000), Ed. Serdi

4. Robert L,
Le Vieillissement. De l'homme à la cellule.
(1994) Ed.CNRS-Belin, Paris

5. Robert L, Labat-Robert J,
Modification of the extracellular matrix of skin during ageing and exposure.
Bioeng. Skin, **4** (1988) 83-85.

6. Labat-Robert J., and Robert L.
Interaction between cells and extracellular matrix : signaling by integrins and the elastin-laminin receptor, In : Progress in molecular and subcellular biology, A. Macieira-Coelho (Ed**25** (2000) 57-70, Springer Verlag, Berlin.

7. Hayflick L,
The cellular basis for biological aging. In: Finch CE, Hayflick L, Eds, Handbook of the Biology of aging. (1977) 159-188, Van Nostrand Reinhold Co, New York

8. Faragher RGA, Jones CJ, Kipling D,
Telomerase and cellular lifespan: Ending the debate?
Nature Biotechnology **16** (1998) 701-702

9. Harley CB, Futcher AB, Greider CW, Telomeres shorten during aging of human fibroblasts. *Nature,* **345** (1990) 548-560

10. Branchet MC, Boisnic S, Francès C, Robert AM,
Skin thickness changes in normal aging skin.
Gerontology. **36** (1990) 28-35.

11. Labat-Robert J, Kern P, Robert L,
Biomarkers of connective tissue aging : biosynthesis of fibronectin, collagen type III and elastase,
Ann. New York. Acad. Sci., **673** (1992) 16-22.

12. Labat-Robert J, Fourtanier A, Boyer-Lafargue B, Robert L,
Age-dependent increase of elastase type protease activity in mouse skin. Effect of UV-irradiation,
J. Photochem. Photobiol. **57** (2000) 113-118

13. Herrling T, Zastrow L, Fuchs J, Groth N,
Electron spin resonance detection of UVA-induced free radicals
Skin Pharmacol Appl Skin Physiol **15** (2002) 381-383

14. Mockett RJ, Sohal RS,
Oxidative stress may be a causal factor in senescence of animals
In : J-M Robine et al. Eds, Longevity : to the limits and beyond. (1997) 139-154
Springer Verlag, Berlin

15. Hannigan BM, Richardson S-A M, McKenna G,

DNA damage in mammalian cell lines with different antioxidant levels and DNA repair capacities
In : Emerit I,Chance B, Eds, Free Radicals and Aging, (1992) 247-250, Birkhäuser Verlag, Basel

16. Deguine V, Menasche M, Ferrari M, Fraisse L, Pouliquen Y, Robert L,
Free radical depolymerisation of hyaluronan by Maillard reaction products. Role in liquefaction of aging vitreous. *Int. J. Biol. Macromol.*, **22** (1998) 17-22.

17. Deguine V, Menasche M, Fraisse L, Ferrari M, Pouliquen Y, Robert L,
Determination of extracellular matrix degradation by free radicals using viscosity measurement of hyaluronan,
Clin. Chim. Acta, **262** (1997) 147-152.

18. Laurent TC et al, Eds, The Chemistry, Biology and Medical applications of Hyaluronan and its Derivatives. (1998) Portland Press Ltd, London

19. Hyaluronan, Vol.1-2, Kennedy JF et al. Eds, Chemical, Biochemical and Biological Aspects, (2002) Woodhead Publishing Limited

20. Robert L, Labat-Robert J,
Morphogenesis, aging and repair of connective tissues.
Facial Plastic Surgery, **6** (1989) 1-7.

21. Robert L,
Interaction between cells and elastin, the elastin receptor. For the 80th birthday of Ines Mandl
Connective Tissue Research **40** (1999) 75-82

22. Péterszegi G, Texier S, Robert L,
Cell death by overload of the elastin-laminin receptor on human activated lymphocytes: protection by lactose and melibiose.
Eur.J.Clin.Invest. **29** (1999) 166-172

23. Robert L,
Mechanisms of aging of the extracellular matrix.Role of the elastin-laminin receptor. Novartis Price Lecture. *Gerontology,* **44** (1998) 307-317

24. Ghuysen-Itard AF, Robert L, Gourlet B, Berr C, Jacob MP, Loss of calcium-homeostatic mechanisms in polymorphonuclear leukocytes of demented and non demented elderly subjects. *Gerontol.*, **39** (1993) 163-169.

25. Robert L, Le vieillissement. Faits et théories. DOMINOS (1995) Flammarion

26. Labat-Robert J, Robert L, Regulation of the biosynthesis of extracellular matrix macro- molecules with special reference to fibronectin and elastin, *Conn. Tiss. Res.,* **15** (1986) 17-21.
27. Rasoamanantena P, Thweatt R, Labat-Robert J, Goldstein S,
Altered regulation of fibronectin gene expression in Werner syndrome fibroblasts, *Exper. Cell. Res.*, **213** (1994) 121-127.

28. Robert C, Blanc M, Lesty C, Dikstein S, Robert L, Study of skin aging as a function of social and professional conditions: modification of the rheological parameters measured with a non-invasive method – indentometry. *Gerontol.,* **34** (1988) 284-290.

29. Vörös E, Robert C, Robert AM, Comparative study of rheological and surface pattern parameters of human skin as a function of age, work conditions and sun exposure,
Arch. Gerontol. Geriat., **17** (1993)131-142.

30. Péterszegi G, Dagonet FB, Labat-Robert J, Robert L, Inhibition of cell proliferation and fibronectin biosynthesis by Na ascorbate, *Eur.J.Clin.Invest,* **32** (2002) 372-380

31. Péterszegi G, Robert AM, Robert L, Protection by L-fucose and fucose-rich polysaccharides against ROS-produced cell death in presence of ascorbate, *Biomed & Pharmacother* special issue on free radicals

32. Isnard N, Fodil I, Robert L, Renard G, Modulation of cell phenotype during in vitro aging. Glycosaminoglycan biosynthesis by skin fibroblasts and corneal keratocytes, *Experimental Gerontology,* **37** (2002) 1377-1385

33. Robert L, Fodil-Bourahla I, Bizbiz L, Robert AM, Effects of L-fucose and fucose-rich oligo- and polysaccharides (FROP-s) on collagen biosynthesis by human skin fibroblasts. Modulation of the effect of retinol, ascorbate and α-tocopherol.*Biomed & Pharmacother,*
in print

34. Isnard N, Fodil-Bourahla I, Robert AM, Robert L, Stimulation of glycosaminoglycan biosynthesis by L-fucose and fucose-rich polysaccharides. *Biomed & Pharmacother* in print

35. Robert L, Fodil-Bourahla I, Bizbiz L, Péterszegi G, Robert AM, Robert L, Effect of L-fucose and fucose-rich polysaccharides on elastin biosynthesis, in vivo and in vitro, *Biomed & Pharmacother,* this issue

36. Robert L, Robert AM, Elastin, elastase and atherosclerosis, In: Robert AM, Robert L, Eds, *Frontiers of Matrix Biology* **8** (1980) 130-173, Karger, Basel

37. Robert C, Robert AM, Robert L, Effect of a fucose-rich polysaccharide (FROP-3) on skin aging. Indentometric studies on human voluntaries. In preparation

38. Robert L, Robert C, Robert AM, Effect of a fucose-rich polysaccharide (FROP-3) on skin aging. Study of the skin surface microrelief on human voluntaries. In preparation

39. . Robert L, Robert C, Robert AM, Effect of a fucose-rich polysaccharide (FROP-3) on skin aging. Study of the effect on periorbital wrinkles on human voluntaries. In preparation

40. Sève A-P, Hubert J, Bouvier D, Bourgeois C, Midoux P, Roche A-C, Monsigny M, Analysis of sugar-binding sites in mammalian cell nuclei by quantitative flow microfluorometry. *Proc.Natl.Acad.Sci.USA,* **83** (1986) 5997-6001

Table I.

Late effect of FROP-3 preparation. % increase of the two rheological parameters, resistence to pressure (indentation) and elastic rebound, one week after the last application of the preparation. No other treatment was used inbetween.

Designation of voluntaries	Age	% remanence	
		indentation	elasticity
Mal	39	0	20
Mic	50	28	6
Gen	56	13	2
Jos	57	2	6
Mad	68	0	3
	averages:	8,6%	7,4%

Legends for figures

Fig.1a. The most conspicuous sign of skin aging is the loss of skin tissue, of about 7% of the original skin thickness - measured on biopsies - lost every decade. Abscissa: age of persons biopsied, males and females; A. Ordinates: thickness of skin measured by image analysis on histological sections.

Fig.1b. Collagen content of dermis, arbitrary units (10).

Fig.2. Age dependent upregulation of elastase-type proteolytic activity is another factor of skin aging. The lower graph (B) shows the age-dependent increase of elastase activity in mouse-skin extracts (12). The upper graph (A) shows the increase of elastase production by human skin fibroblasts in culture as a function of successive passages (in vitro aging) (11).

Fig.3. Schematic representation of the elastin-laminin-receptor with its 3 subunits and its message-transmission pathway, the G_i-protein, phospholipase C (PLC), as well as the inhibitors used to block the transmission. Elastin peptides are the agonists of this receptor, and FROP-s act as antagonists. In young cells this receptor mediates a number of physiological functions. In old cells there is an uncoupling of the transmission pathway at the level of the G_i protein eliminating several useful functions, but increasing free radical release (see Fig.4.) and elastase release. Two of the free radicals released , superoxide and $NO^{\bullet}$ can combine to form the highly toxic peroxinitrite anion ($ONOO^-$), increasing the harmful effects mediated by the receptor and efficiently inhibited by FROP (6,21,23). This can also be inhibited by reduced glutathion (GSH) which decreases with age.
Below: Demonstration by immunofluorescence (rhodamine) of the presence of the elastin-laminin receptor on human skin fibroblasts.

Fig.4. Superoxide release mediated by the elastin-laminin receptor (●--●; see Fig.3.). In "young" cells this free radical release can be inhibited by pertussis toxin (○--○), an

inhibitor of the G_i protein, as shown on Fig.3. In "old" cells this inhibition becomes inefficient and free radical release is increased (6,21,23).

Fig.5. Demonstration of free radical scavenging by RROP. The viscosity of a hyaluronan solution is recorded in the absence of free radicals and in presence of the Udenfriend reagent releasing $OH^{\bullet}$ radicals, producing a rapid drop of viscosity. This drop of viscosity is inhibited in presence of RROP at low concentrations (1 to10 μg/ml) (17).

Fig.6. Dose-effect curves of the free radical scavenging by RROP and FROP. The former is efficient at low concentrations and reaches than a saturation. FROP-s efficiency is increasing with concentration without reaching a plateau.

Fig.7. Effect of L-fucose and FROP-s on cell-proliferation determined by ^{3}H-thymidine incorporation. % increase of incorporation as compared to control. The stimulating effect of L-fucose was compared to the fucose-rich polysaccharide Fucogel and to three FROP-preparations (1).

Fig.8. Stimulation by RROP of protein and collagen biosynthesis by human skin fibroblasts. Increasing concentrations produce increasing incorporation of ^{3}H-proline in proteins (upper part of graph) and in collagens (lower part of graph).

Fig.9. Stimulation by L-fucose and FROP of total protein and collagen biosynthesis by the incorporation of ^{3}H-proline (33).

Fig.10. Effect of all-trans retinol, of FROP and L-fucose on total protein and collagen biosynthesis, alone or in combination. Notice the strong stimulation by the combined effects of retinol and FROP (33).

Fig.11. Effect of 4 weeks of local treatment with L-fucose or FROP on the thickness of epidermis (I) and dermis (II). A. untreated skin B. treated skin. Notice the strong increase of cells and extracellular matrix.

Fig.12. Increase of collagen fiber density of hairless rat skin after 4 weeks of local treatment with L-fucose or with FROP as shown by the density of the histological sections (left) and by the distribution profile of fiber density evaluated by computerised image analysis (right). The left-shift of the distribution curve indicates the strong increase of fiber density in the treated skin.

Fig.13. Histogram representing the increase of elastin fiber formation in L-fucose or FROP-treated hairless rat skins after 4 weeks of treatment (35).

Fig.14. Effect of FROP-containing topical preparation on the rheological properties of human skin, determined by indentometry on 20 women voluntaries (37). A. increase of resistence to pressure (indentation, hydration); B. increase of elastic rebound.

Fig.15. Histogram showing the effect of the FROP-containing topical preparation on the periorbital wrinkles of 18 voluntary women. The wrinkles are evaluated by the product of total length and average width (wrinkle coefficient) and represented as % of modification after 4 weeks of local treatment (39). + values: improvement in %; - values: deterioration.

Fig.16. Microphotograph showing a human skin fibroblast reacted with fluorescein-marked FROP which reacts specifically with cell-membrane localised receptors and penetrates in the nucleus (1).