In vitro reconstructed tissues on hyaluronan-based temporary scaffolding

P. BRUN, R. CORTIVO, B. ZAVAN, N. VECCHIATO, G. ABATANGELO*
Institute of Histology and Embryology, Faculty of Medicine, University of Padova, Viale G. Colombo, 3, 35121 I-Padova, Italy
E-mail: abatange@civ.bio.unipd.it

Tissue engineering offers the possibility to reconstruct tissue substitutes in order to replace lost or damaged tissues. The availability of appropriate biomaterial devices is essential to allow *in vitro* cultured cells to behave as in the original tissues *in vivo*. In our studies we utilized a seminatural biomaterial made up by the benzyl ester of hyaluronan to grow keratinocytes, fibroblasts and chondrocytes. Keratinocytes and fibroblasts were isolated from human foreskin. Cells were separately cultured on two different hyaluronan based biomaterial devices for the first 15 days and then co-cultured for an additional period of 2 weeks. Keratinocytes gave rise to a well-differentiated epithelial layer, while fibroblasts were able to synthesize all the main extracellular molecules inside the biomaterial spaces, forming dermal-like tissues. When these two tissues were co-cultured, a skin equivalent was formed with a dermal–epidermal junction. Chondrocytes were obtained from chick-embryo sterna and cultured for 21 days inside a non-woven scaffolding made up of a hyaluronan-based biomaterial. Cells were able to organize themselves into nodules embedded in a dense metachromatic substance in which type II collagen was present. Data from this study suggest that this novel class of hyaluronan derived biomaterials is suitable for different cell culture and *in vitro* tissue reconstruction.

1. Introduction

Many diseases cannot simply be managed by pharmacological therapies and therefore they represent severe problems for the clinician. Over the past 80 years, the clinical solution to restoring structure and function of damaged tissues and organs has been based on the search for other therapies with the aim of overcoming this medical gap. The first successful approach in treating such diseases was cell transplant. The infusion of homologous blood cells started the era of the cell therapy. Later on, great improvements made in surgical procedures and in the immunological field allowed the substitution of whole organs, such as kidney, heart, liver, lung, and pancreas, obtained from healthy donors or cadavers. Although these solutions have resulted in high increase of patient survival, the numbers of donors and graft rejection represent the major limiting difficulties for this practice. A new impetus for the development of alternative strategies is represented by cell culture technologies that can offer to scientists the possibility to collect, isolate, cultivate and expand almost all kinds of animal and human cells starting from small biopsies. This new frontier, called tissue engineering, is based on the assumption that tissue cells, when cultured *in vitro*, must behave in a way similar to that of the original tissue. In other words, cells must proliferate, differentiate and organize themselves in order to reconstruct a suitable environment that can allow their original expression. According to this principle, one might hypothesize that the new strategy for applied clinical research should be the reconstruction *in vitro* of tissues and entire organs obtained by guided *in vitro* proliferation of autologous cells of the same patient to be eventually grafted [1]. It is a matter of fact, however, that cells seeded in plastic culture devices expand only in a two-dimensional way. In such a condition cells cannot synthesize and deposit extracellular matrix (ECM) components and, often, undergo dedifferentiation. The lack of a three-dimensional architecture and the absence or scarcity of specific ECM components, which are essential for regulating cellular activities, may cause the loss of the original phenotype of the cultured cells. To overcome these problems particular medical devices have been proposed as scaffolding for the guided cell growth *in vitro*. In this way, cells can expand in a three-dimensional support in which they can deposit a surrounding ECM [2]. As a final result, an engineered tissue is obtained which shares similar properties with the native tissue and can be utilized *in vivo* as a tissue substitute. Currently, various kinds of biomaterials are available for cell culture and tissue reconstruction which can be roughly classified in three main categories: natural, semisynthetic (or seminatural) and synthetic. Many of these have been tested for their biocompatibility and biodegradability both *in vitro* and *in vivo* [3–9]. Protein-based polymers such as

*Author to whom correspondence should be addressed.

collagen, fibronectin, fibrin have been chemically modified to form materials that can be molded in various shapes. Glycosamino-glycans (GAGs), such as hyaluronan, chondroitin sulfate, dermatan sulfate and chitin, have also been covalently modified to alter their water solubility and degradation rates. These modified natural substances represent the semisynthetic biomaterials which are widely used as scaffolding for cell culture and cell delivery. Recently, hyaluronan, a non-sulfated glycosaminoglycan has been modified by the esterification of the carboxyl groups along the backbone with aliphatic or aromatic alcohols to form a water insoluble biomaterial which can be processed to produce spun fibers, woven and non-woven textiles, meshes, membranes, etc. Hyaluronan is a ubiquitous molecule present in all the connective tissues and it has been shown to exert a fundamental role in many biological processes such as water balance, cell recognition, embryonic development [10–22] and wound healing both in adult and fetal life stages [23–27]. When considering all these biological properties, for many researchers it has been tempting to think that hyaluronan derivatives may constitute suitable supports for the growth of mammalian cells [28–32]. In particular, mature cells or stem elements can be stimulated to divide and differentiate in contact with this natural non-sulfated polysaccharide [33–34]. In our laboratories we have tested various formulations of esterified hyaluronan to assess their biocompatibility and biodegradability [35, 36] and the most suitable devices for cell culture were found to be represented by the benzyl ester of hyaluronan, called HYAFF-11® (Fidia Advanced BioPolymers; FAB, Abano T., Italy). Two different forms of this biomaterial were used: non-woven meshes (20-μm-thick fibers, with a specific weight of $100\,g\,m^{-2}$) and microperforated membranes (20-μm-thick membranes with laser-made micropores of 40 μm in diameter, LaserSkin™). In the present work we studied the behavior of various cell types cultured on these hyaluronan derived supports, such as keratinocytes, fibroblasts and chick embryo chondrocytes.

2. Materials and methods

2.1. Biomaterials

Biomaterials used in the present study were both derived from the total esterification of hyaluronan with benzyl alcohol [37, 38] and referred to as HYAFF-11® (100% nominal degree of substitution, effective range was 92–100%). The molecule is synthesized starting from 80–200 kDa sodium hyaluronate. The final product is non-cross-linked linear polymer with an undetermined molecular weight and insoluble in aqueous solutions, even though it spontaneously hydrolyses with time, releasing benzylic alcohol and hyaluronan.

Two different forms of HYAFF-11 were used: (1) non-woven meshes made of 20-μm-thick fibers, with a specific weight of $100\,g\,m^{-2}$; (2) 20-μm-thick membranes with laser-made micropores of 40 μm in diameter (LaserSkin®). These devices were obtained from Fidia Advanced Biopolymers (FAB, Abano T., Italy).

2.2. Cell cultures

2.2.1. Fibroblast–keratinocyte composite cultures

Keratinocytes and fibroblasts were isolated from human foreskin. Keratinocytes were obtained from epidermis by trypsin digestion, while fibroblasts were isolated from dermis by sequential trypsin and collagenase digestion. Keratinocytes were cultivated according to the method of Rheinwald and Green [39, 40] and fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, L-glutamine (4 mM), and penicillin–streptomycin $(50\,i\,ml)^{-1}$ (all from Seromed, Berlin, Germany). At confluence fibroblasts were harvested and seeded at a density of $3 \times 10^5\,cm^{-2}$ on squares of non-woven meshes (1.5×1.5 cm) in the above-mentioned medium containing sodium ascorbate ($50\,\mu g\,ml^{-1}$). The non-woven squares had been fixed on culture plates by mean either of stainless steel rings or with a fibrin clot. Keratinocytes were plated in secondary cultures at a density of 2×10^4 cells cm^{-2} onto the LaserSkin membrane in the presence of a feeder layer of non-proliferating Swiss BALB/c 3T3 mouse fibroblasts. At confluence (10–12 days), pieces of membranes (1.5×1.5 cm) were laid down and fixed to the non-woven meshes on which dermal fibroblasts had been grown for 15 days. The fibroblast–keratinocyte composite cultures were laid down carefully on sterile stainless steel grids and then cultivated for 15 days at the air–liquid interface. At the end of this period, cultures were collected without using proteolytic enzymes and gently cut in two halves: one was fixed in 10% buffered formalin and, after paraffin embedding, processed for routine histology. The other was embedded in OCT (Tissue-Tek, Elkhart, IN) and immediately deep-frozen to be used for immunohistochemical characterization.

2.2.2. Chick-embryo chondrocytes

Primary cultures of chick embryo chondrocytes were established, as described elsewhere [41–43]. Briefly, 15-day-old chick embryos were sacrificed and the sterna removed in aseptic conditions. Each sternum was then finely minced and pooled biopsies were dissolved by serial enzymatic digestions in trypsin (Gibco), testicular hyaluronidase (Sigma, St. Louis, MO) and collagenase type I (Sigma), respectively. Finally, digested material was centrifuged at 1000 r.p.m. for 10 min, and pellets resuspended in complete medium (Ham's F12 supplemented with 10% fetal calf serum (FCS), 1% pennicillin/streptomycin, 1% L-glutamine, $50\,\mu g\,ml^{-1}$ of L-ascorbic acid (Sigma), $1\,ng\,ml^{-1}$ of transforming growth factor-β1 (TGF-β1 Calbiochem), $1\,ng\,ml^{-1}$ of insulin (Sigma), 1 ng ml epidermal growth factor (EGF; Sigma) and $10\,ng\,ml^{-1}$ of b-fibroblast growth factor (bFGF) (Calbiochem). Viability of each preparation was checked by trypan blue exclusion. Medium was changed every other day and cells were routinely expanded on plastic. Only cells at the first or second passage were used for experiments. Chick chondrocytes were trypsinized, detached from the plastic flasks and seeded onto the hyaluronan-derived non-woven fleeces (1×1 cm). The biomaterials were completely hydrated with approxi-

mately 100–150 µl of medium containing 2×10^5 cells. Cultures were then returned to the incubator and, after 3 h, 1.5 ml of complete medium were added to each capsule. The same cell density was used to set up controls on 35-mm diameter coverslips placed in Petri dishes. At 1, 2, and 3 weeks after seeding the biomaterials were harvested and cut in two: one half snap-frozen for histology and the other processed for electron microscopy. Histological analyses were also performed on the control chondrocytes grown on sterilized coverslips. Snap-frozen biomaterial scaffolding were sliced into sections of 5 µm, placed on gelatin-coated slides and fixed with acetone for 10 min at room temperature. Samples were stained with 1% Toluidine blue (pH 2.0) for histochemistry. For immunohistochemistry, sections were stained with a mouse antichick collagen type II monoclonal antibody, obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Immune reaction was revealed by the avidin–biotin system using Vectstain-ABC kit (Vector Laboratories, Burlingame, CA) and slides were stained with 3-amino-9-ethylcarbazole and were counterstained with haematoxylin. Positive control histological sections obtained from chick embryo sternum were treated for 30 min at 37 °C with bovine 5 mg ml^{-1} testicular hyaluronidase (Sigma) in PBS at pH 6.0 before immunostaining for collagen type II. Negative control sections were obtained by omitting the primary antibody. This same staining procedure was applied to the control chondrocytes grown on coverslip.

All cell cultures were done in triplicate with three different cell preparations and the histological pictures are representative of all the different samples.

2.3. Proliferation assay

When needed, the proliferation rate of cells within the non-woven mesh was determined by the 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) based cytotoxicity test, performed according to the method of Denizot and Lang [44] with minor modifications. The test is based on mitochondrial viability, i.e. only functional mitochondria can oxidize MTT solution giving a typical blue–violet end-product. This assay is an indirect method to assess cell growth and proliferation since the OD values can be correlated to the cell number.

At fixed intervals, non-woven pieces were detached from the culture dishes and transferred into new wells and MTT was added to establish cell growth. Sister cultures in the absence of biomaterial were used as controls.

3. Results

3.1. Fibroblast–keratinocyte co-cultures

Human fibroblasts seeded onto non-woven HYAFF-11 were able to adhere to the textile fibers and proliferate. Fibroblast cultures were monitored for up to 30 days, after which the biomaterial began to dissolve into the medium. As demonstrated previously [32], during the first week of cell growth, fibroblast proliferation into the biomaterial non-woven mesh was comparable to that of the control culture dishes. In later stages of culture, while fibroblasts seeded in culture plates reached confluence after 1 week and then significantly reduced their proliferation, cells seeded onto non-woven mesh continued to proliferate because they had the possibility to spread in three-dimensional manner, filling the scaffolding spaces [32].

Keratinocytes seeded onto LaserSkin membranes proliferated and reached confluency after 10–12 days. They colonized both sides of the membrane, migrating through the micropores (Fig. 1). When these membranes were detached from the dish and placed on the top of the non-woven meshes in which human fibroblasts had been cultivated for 2 weeks the composite cultures formed, after an additional 2 weeks, an artificial skin consisting of both a well-differentiated epithelial layer and a dermal-like structure. Fig. 2 shows the histological appearance of a composite culture of keratinocytes and fibroblasts. On the top of the figure an epidermal layer of differentiated keratinocytes is clearly evident, while fibroblasts are present in the underlying non-woven mesh. The immunohistochemical analyses of the main differentiation markers of keratinocytes, basal lamina and dermal equivalent tissue are reported in Table I. It can be seen that keratinocytes of the superficial layers express keratin, involucrin and filagrrin, while keratinocytes facing the dermal-like tissue express β-1 and β-4 integrin subunits. Fibronectin, laminin-1, type I, III and IV

Figure 1 Cross-section of keratinocyte-loaded microperforated membrane (m) at 15 days of culture. On the upper side there is a pluristratified layer of terminally differentiated keratinocytes and the cells that have migrated through the holes have formed a well stratified epithelium beneath the membrane. 1% Toluidine Blue staining (200×).

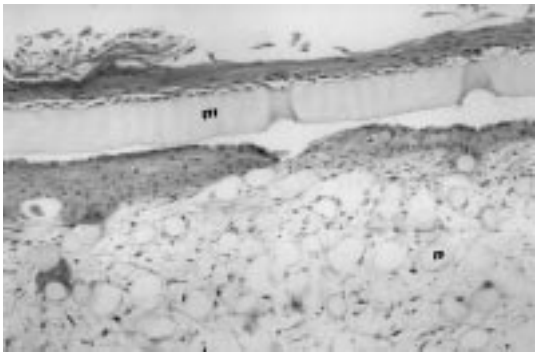

Figure 2 Cross-section of a fibroblast-keratinocyte co-culture after 15 days. In the upper part of the figure keratinocytes present on both sides of the HYAFF membrane (m) are clearly distinguishable. The lower part of the figure shows the dermal-like structure in which fibroblasts are randomly dispersed around the fibers of the non-woven mesh (n). The keratinocyte layer facing the dermal-like structure present a region of proliferating cuboidal-shaped cells in contact with the extracellular matrix secreted by fibroblasts present in the underlying non-woven mesh (haematoxylin and eosin; 100×).

TABLE I Expression of differentiation markers in keratinocyte-fibroblast co-culture

	Involucrin filaggrin keratin	β1 and − β4 integrin subunits	Type I and III collagen	Type IV collagen fibronectin laminin-1	Type VII collagen laminin-5
Suprabasal KC	+	−	−	−	−
Basal KC	−	+	−	−	−
Basal lamina	−	−	−	+	+
Dermal-like layer	−	−	+	+	−

+ Marker expressed; – marker unexpressed.
KC, keratinocytes.

collagens are present in the dermal-like layer. The specific molecules of the basal lamina, such as type VII collagen and laminin-5 are expressed at the epidermal-dermal junction.

3.2. Chick-embro condrocytes

Chondrocytes seeded onto non-woven fleeces of the biomaterial adhered in a few hours. With phase contrast microscopy, cells cultivated on hyaluronan-based scaffolding appeared round in shape and most chondrocytes seemed to adhere to the fibers 3 h after seeding (data not shown).

Toluidine Blue staining of sections showed that the chondrocytes tended to form nodules of different sizes within one week of culture. The chondrocyte clusters formed in the non-woven interstices, appear embedded in an apparently GA G rich, dense positive metachromatic substance, as shown in Fig. 3, where a semithin section after Toluidine Blue staining is represented. Cells at the periphery of the nodules appear flattened, while chondrocytes in the inner part of the cluster were large and ovoid. In the biomaterial scaffolds the cartilage nodules at weeks 2 and 3 consistently demonstrated an abundant metachromatic extracellular matrix. In contrast, chondrocytes grown on glass coverslips progressively assumed a fibroblast-like appearance with a few interspersed clusters of round cells surrounded and embedded in a metachromatic substance which after 2 weeks tended to completely disappear (data not shown). The immunolocalization of type II collagen around the cartilage-like nodules formed inside the biomaterial scaffolding confirmed the chondrocyte phenotype which firmly persisted up to three weeks (Fig. 4). Control chondrocytes grown on coverslips were able to synthesize type II collagen only during the first week of culture and then its presence progressively decreased through the second week to disappear completely after three weeks (data not shown).

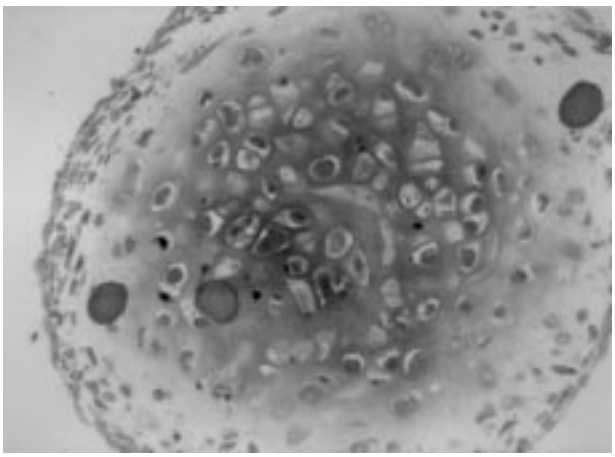

Figure 3 Cross-section of 3 weeks culture of chick embryo chondrocytes grown onto hyaluronan based fleeces and stained with 1% Toluidine Blue. The central part of the cartilage nodule is populated by round shaped cells, while the periphery is characterized by the presence of flattened and elongated cells. The metachromatic staining, which is evident in the central part of the chondrocyte cluster, stands for glycosaminolycans deposition around the cells. (1% Toluidine Blue staining; 200 ×).

4. Discussion

In this paper we have described the use of semisynthetic scaffolding made up by the benzyl ester of hyaluronan (Hyaff materials) in which keratinocyte, fibroblasts and chondrocytes were cultured.

4.1. Keratinocytes and fibroblasts

Keratinocytes grown on LaserSkin membranes gave rise to a well-differentiated stratified epithelium, while fibroblasts were able to populate a three-dimensional non-woven mesh of HYAFF-11 and deposit in the interfibrillar spaces extracellular matrix, forming a dermal-like tissue. It has been possible to demonstrate that, with a composite culture system, cultured keratinocyte sheets could be placed onto the non-woven mesh in which fibroblasts had been cultured for 15 days. After an

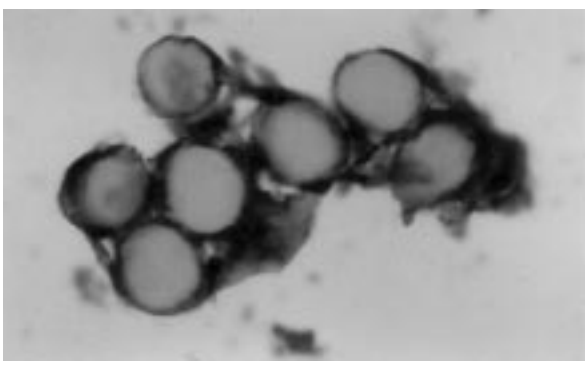

Figure 4 Immunolocalization of type II collagen (*dark staining*) in chick embryo chondrocyte cluster after 3 weeks from seeding into HYAFF non-woven mesh. Counterstaining with haematoxylin. (400×).

additional 2 weeks, the composite cultures formed a skin-like tissue consisting of both a well differentiated epithelial layer and a dermal-like structure. As expected, the interaction of these two distinct tissues gave rise to an evident basal lamina at the interface between keratinocytes and fibroblasts.

Mesenchymal–epithelial interactions have been demonstrated to regulate several aspects of the epithelial development and homeostasis [45]. In fact, much evidence supported the concept that the dermal component constitutes a permissive and regulatory microenvironment for the growth and differentiation of cultured keratinocyte sheets [46, 47]. Composite co-cultures of both fibroblasts and keratinocytes have improved clinical outcomes of skin transplants [49–57], and the possible future use of this organotypic culture as *in vitro* cytotoxicity assay for the replacement of current animal models is strongly encouraged [58]. In clinical practice, even though keratinocyte culture techniques have improved, the use of keratinocyte laminae for wound treatment is often unsatisfactory [53]. Indeed, it is now widely accepted that, in the presence of a dermal support, cultured keratinocyte grafts might overcome several problems arising from their clinical application, such as fragility, difficult handling and contraction. In addition, the clinical use of composite fibroblast/keratinocyte cultures may definitely improve the take-rate at wound sites.

4.2. Chondrocytes

Chondrocytes seeded onto three-dimensional scaffolding of HYAFF materials diffused into the fibril network and adhered to the biomaterial fleeces. Clusters of chondrocytes around the biomaterial fibers were observed directly with phase contrast microscopy after 3 h of culture. Cells then started to proliferate and those trapped in the interstices of the scaffolding were able to form cartilage nodules of different sizes. All the nodules become embedded in their own matrix made up of a dense metachromatic substance, rich in glycosaminoglycans and type II collagen. The presence of the latter demonstrated that the chondrocyte cultured inside these hyaluronan-based biomaterials retained their phenotype, since they were able to synthesize collagen type II, a typical marker for hyaline cartilaginous ECM. In contrast, the control chondrocytes cultured on coverslips formed a monolayer of cells, the morphology of which was quite different from that observed in the cultures inside the biomaterial scaffolding. After 1 week of culture, chondrocytes grown on glass or plastic surfaces assumed a fibroblast-like appearance. Only scattered clusters of round cells could be observed within a Toluidine Blue-positive material, the chondrocyte phenotype of which was confirmed by the presence of collagen type II. It is reasonable to speculate that, as in native cartilage, the presence of hyaluronan, a naturally occurring non-sulfated glycosaminoglycan, is crucial for chondrocyte growth and organization, since they come in contact with a ''natural'' substrate, in which cells grow in a three-dimensional tissue-like structure.

The clinical use of cultured chondrocytes in an *in vitro* reconstructed tissue is a potentially powerful tool for the treatment of cartilage defects. As known, the poor regenerative capacity of articular mammalian cartilage and the lack of therapeutically effective drugs lead to progressive degenerative changes of this hard connective tissue [59]. Recently, some research groups have begun to draw their attention to cell therapy for the treatment of a small variety of joint cartilage pathologies [60, 61]. In these cases, autologous chondrocytes were harvested arthroscopically and, after *in vitro* expansion, were grafted as a suspension to the patient's defect and secured *in situ* by means of a periosteal flap. Although data from these and other reports are encouraging, some researchers are still sceptical of the efficacy of this technique [62]. Instead, cultured chondrocyte nodules, embedded in their own extracellular matrix synthesized inside a biocompatible temporary scaffolding, such as those used in the present work, should have better handling properties for the end-user, as cells are not in suspension but entrapped in a biocompatible and resorbable scaffolding.

In this perspective, our results represent a preliminary encouraging step towards the clinical implementation of autologous chondrocyte grafting for the treatment of cartilage lesions.

References

1. R. LANGER and J. P. VACANTI, *Science* **260** (1993) 920.
2. I. V. YANNAS, *J. Cell Biochem.* **56** (1994) 188.
3. S. SRIVASTAVA, S. D. GORHAM, D. A. FRENCH, A. A. SHIVAS and J. M. COURTNEY, *Biomaterials* **11** (1990) 155.
4. M. L. COOPER, J. F. HANSBROUGH, R. L. SPIELVOGEL, R. COHEN, R. L. BARTEL and G. NAUGHTON, *ibid.* **12** (1991) 243.
5. A. W. BLAYNEY, K. R. WILLIAMS, J. P. ERRE, T. H. LESSER and M. PORTMANN, *Acta Otolaryngol. Stockh.* **112** (1992) 322.
6. G. J. BEUMER, C. A. VAN BLITTERSWIJK, D. BAKKER and M. PONEC, *Biomaterials* **14** (1993) 598.
7. R. B. BUSH, *J. Biomed. Mater. Res.* **33** (1996) 107.
8. E. MATOUSKOVA, D. VOGTOVA and R. KONIGOVA, *Burns* **19** (1993) 118.
9. H. O. RENNEKAMPFF, V. KIESSIG and J. F. HANSBROUGH, *J. Surg. Res.* **62** (1996b) 288.
10. G. ABATANGELO, P. BRUN and R. CORTIVO, in ''Novel Biomaterials based on Hyaluronic acid and its derivatives'', Proceedings of a Workshop held at the Annual Meeting of the European Society for Biomaterials, Pisa, Italy, 1994, edited by D. F. Williams (University Press, Liverpool, UK) p. 8.
11. B. P. TOOLE, S. I. MUNAIM, S. WELLES and C. B. KNUDSON, in ''CIBA Foundation Symposium 143, The biology of hyaluronan'' (Wiley, Chichester, 1989) p. 138.
12. B. P. TOOLE and R. L. TRELSTAD, *Dev. Biol.* **26** (1971) 28.
13. M. P. MALESKI and C. B. KNUDSON, *Conn. Tissue Res.* **34** (1996) 75.
14. B. A. MAST, R. F. DIEGELMANN, T. M. KRUMMEL and I. K. COHEN, *Matrix* **13** (1993) 441.
15. P. ROONEY and S. KUMAR, *Differentiation* **54** (1993) 1.
16. W. GOTZ, R. OSMERS and R. HERKEN, *J. Anat.* **186** (1995) 111.
17. A. A. PITSILLIDES, C. W. ARCHER, P. PREHM, M. T. BAYLISS and J. C. EDWARDS, *J. Histochem. Cytochem.* **43** (1995) 263.
18. S. C. WHEATLEY, C. M. ISACKE and P. H. CROSSLEY, *Development* **119** (1993) 295.
19. J. J. BROWN and V. E. PAPAIOANNOU, *ibid.* **117** (1993) 483.
20. V. KRENN, B. BRAND-SABERI and F. WACHTLER, *Amos. J. Anat.* **192** (1991) 400.
21. P. E. PETERSON, C. S. POW, D. B. WILSON and A. G. HENDRICKX, *Acta Anat. Basel* **146** (1993) 13.
22. M. J. KUJAWA and A. I. CAPLAN, *Dev. Biol.* **114** (1986) 504.

23. K. P. MORIARTY, T. M. CROMBLEHOLME, E. K. GALLIVAN and C. O'DONNELL, *Wound Rep. Reg.* **4** (1996) 346.
24. C. B. KNUDSON and W. KNUDSON, *FASEB J.* **7** (1993) 233.
25. *Idem.*, *Cancer Lett.* **52** (1990) 113.
26. O. OKSALA, T. SALO, R. TAMMI, L. HAKKINEN, M. JALKANEN, P. INKI and H. LARJAVA, *J. Histochem. Cytochem.* **43** (1995) 125.
27. J. W. SIEBERT, A. R. BURD, J. G. MCCARTHY, J. WEINZWEIG and H. P. EHRLICH, *Plast. Reconstr. Surg.* **85** (1990) 495.
28. P. GIUSTI and L. CALLEGARO, World Patent Application NoWO 94/01468, 1994.
29. T. W. SANDER and D. S. KAPLAN, US Patent NO 5,356,629, 1994.
30. K. C. SUNG and E. M. TOPP, *J. Membrane Sci.* **92** (1994) 157.
31. F. DELLA VALLE and A. ROMEO, US Patent No 5,466,461, 1995.
32. V. ZACCHI, C. SORANZO, R. CORTIVO, M. RADICE, P. BRUN and G. ABATANGELO, *J. Biomed. Mater. Res.* **40** (1998) 186.
33. R. CORTIVO, A. DE GALATEO, I. CASTELLANI, P. BRUN, M. G. GIRO and G. ABATANGELO, *Cell Biol. Int. Rep.* **14** (1990) 111.
34. S. SHEPARD, H. BECKER and J. X. HARTMANN, *Ann. Plast. Surg.* **36** (1996) 65.
35. R. CORTIVO, P. BRUN, A. RASTRELLI and G. ABATANGELO, *Biomaterials* **12** (1991) 727.
36. L. BENEDETTI, R. CORTIVO, T. BERTI, F. PEA, M. MAZZO, M. MORAS and G. ABATANGELO, *ibid.* **14** (1993) 1154.
37. F. DELLA VALLE and A. ROMEO, Eur. Patent Appl. EP 216453, (1987) p. 129.
38. A. RASTRELLI, M. BECCARO, F. BIVIANO, G. CALDERINI and A. PASTORELLO, in ''Clinical implant materials, advances in biomaterials'', Vol. 9, edited by G. Heimke, U. Soltész and A. J. C. Lee (Elsevier Science Publishers, Amsterdam, The Netherlands 1990) p. 199.
39. J. G. RHEINWALD and H. GREEN, *Cell* **6** (1975) 317.
40. *Idem.*, *ibid.* **6** (1975) 331.
41. R. CORTIVO, A. DE GALATEO, I. CASTELLANI, P. BRUN, M. G. GIRO and G. ABATANGELO, *Cell. Biol. Int. Rep.* **14** (1990) 111.
42. A. I. CAPLAN, *J. Orthop. Res.* **9** (1991) 641.
43. *Idem.*, *Bone Rep. Reg.* **21** (1994) 429.
44. F. DENIZOT and R. LANG, *J. Immunol. Methods* **89** (1986) 271.
45. I. MACKENZIE, in ''The Keratinocyte handbook'', edited by I. Leigh, B. Lane and F. M. Watt (Cambridge University Press, Cambridge, 1994) p. 243.
46. B. COULOMB, C. LEBRETON and L. DUBERTRET, *J. Invest. Dermatol.* **92** (1989) 122.
47. N. C. KREJCI, C. B. CUONO, R. C. LANGDON and J. MCGUIRE, *ibid.* **97** (1991) 843.
48. N. E. FUSENIG, in ''The Keratinocyte handbook'' edited by I. Leigh, B. Lane and F. M. Watt (Cambridge University Press, Cambridge, 1994) p. 71.
49. L. DUBERTRET and B. COULOMB, *Bull. Acad. Natl Med.* **179** (1995) 1121.
50. S. T. BOYCE, M. J. GORETSKY, D. G. GREENHALGH, R. J. KAGAN, M. T. RIEMAN and G. D. WARDEN, *Ann. Surg.* **222** (1995) 743.
51. M. L. COOPER and J. F. HANSBROUGH, *J. Cell Biochem.* **14E** (1990) 251.
52. C. B. CUONO, R. C. LANGDON, N. BIRCHALL, S. BARTTELBORT and J. MCGUIRE, *Plast. Reconstr. Surg.* **80** (1987) 626.
53. J. F. HANSBROUGH, J. MORGAN and G. GREENLEAF, *Wounds* **5** (1993) 174.
54. E. HECK, P. BERGSTRESSER and C. BAXTER, *J. Trauma* **25** (1985) 106.
55. D. A. MEDALIE, S. A. EMING, R. G. TOMPKINS, M. L. YARMUSH, G. G. KRUEGER and J. R. MORGAN, *J. Invest. Dermatol.* **107** (1996) 121.
56. H. O. RENNEKAMPFF, J. F. HANSBROUGH, V. KIESSIG, S. ABIEZZI and V. WOODS JR, *Surgery* **120** (1996) 16.
57. M. L. SABOLINSKI, O. ALVAREZ, M. AULETTA, G. MULDER and N. L. PARENTEAU, *Biomaterials* **17** (1996) 311.
58. F. H. M. PISTOOR, A. RAMBUKKANA, M. KROEZEN, J. P. LEPOITTEVIN, J. D. BOS, M. L. KAPSENBERG and P. K. DAS, *Am. J. Pathol.* **149** (1996) 337.
59. M. METSÄRANTA, U. M. KUJALA, L. PELLINIEMI, H. ÖSTERMAN, H. AHO and E. VUORIO, *Matrix Biol.* **15** (1996) 39.
60. T. MINAS and S. NEHRER, *Orthopedics* **20** (1997) 525.
61. M. BRITTBERG, A. LINDAHL, A. NILSSON, C. OHLSSON, O. ISAKSSON and L. PETERSON, *N. Engl. J. Med.* **331** (1994) 889.
62. K. MESSNER and J. GILLQUIST, *Acta Orthop. Scand.* **67** (1996) 523.

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