Biocompatibility of collagen membranes crosslinked with glutaraldehyde or diphenylphosphoryl azide: An *in vitro* study

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Abstract: Crosslinking of collagen biomaterials increases their resistance to degradation *in vivo*. Glutaraldehyde (GA) is normally used to crosslink collagen biomaterial, but is often cytotoxic. Diphenylphosphoryl azide (DPPA) has recently been proposed as reagent, but little is known about its effects on cell behavior. In this study, we determined which collagen membrane was the most biocompatible: Paroguide which is crosslinked with DPPA and contains chondroitin sulfate; Opocrin which is crosslinked with DPPA; Biomed Extend which is crosslinked with GA; and Bio-Gide which is left untreated. Cell proliferation and extracellular matrix macromolecule deposition were evaluated in human fibroblasts cultured on the membranes. The GA-crosslinked Biomed Extend membrane and the not-crosslinked Bio-Gide membrane reduced cell growth and collagen secretion compared with DPPA-crosslinked biomembranes. When Paroguide and Opocrin were compared, better results were obtained with Paroguide. The greatest amount of transforming growth factor β_1, a growth factor involved in extracellular matrix macromolecule accumulation and in tissue regeneration, was produced by cells cultured on Paroguide, with Opocrin second. Our data suggest that the DPPA method is more biocompatible than the GA for crosslinking collagen biomaterials and that membranes made of collagen plus chondroitin sulfate are better than membranes made of pure collagen. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 67A: 504–509, 2003

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INTRODUCTION

Guided tissue regeneration procedures currently use occlusive barrier membranes to encourage the formation of bone, the periodontal ligament, and the cementum around roots of teeth, when these tissues have been lost because of periodontal disease. Membranes function as mechanical barriers. They exclude epithelium and gingival corium from the root surface and allow bone regeneration. Bioabsorbable collagen membranes have become a popular biomaterial because they support regeneration, preclude the need for surgical removal, allow cell adherence, stimulate cell migration, and support cell proliferation, particularly epidermal cells and fibroblasts.[1,2] Moreover, they show naturally low antigenicity and high biocompatibility.

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Collagen materials for long-term biomedical applications need to be submitted to crosslinking to improve the mechanical stability of the biopolymer and induce controlled biodegradability because, when not crosslinked, collagen membranes exhibit poor mechanical properties and rapid degradation that limits their application as a biomaterial.[3] Glutaraldehyde (GA) is the most widely used chemical crosslinking agent because it stabilizes collagen efficiently.[3] However, GA-crosslinked biomaterials are poorly biocompatible with some cell lines including human fibroblasts, osteoblasts, Chang cells, and endothelial cells.[4–6] The side effects of GA treatment are, at least in part, attributed to the degradation of the GA-derived crosslinks and to the continual release of aldehydes that contribute to prolonged toxic effects.[4,7]

To overcome these disadvantages, alternative crosslinking with diphenylphosphoryl azide (DPPA) was developed.[8] Crosslinking with DPPA increases resistance to degradation of collagen biomaterial and seems to be more biocompatible than crosslinking with GA.[3,9]

In this *in vitro* study, we evaluated the biocompatibility of collagen membranes that were left untreated or crosslinked with GA or DPPA methods. We attempted to clarify the effects of crosslinking agents by studying cell growth and the synthesis of extracellular matrix (ECM) macromolecules such as collagen and glycosaminoglycan (GAG). Neosynthesis of ECM macromolecules was analyzed by labeled precursor incorporation. Secreted transforming growth factor (TGF)β_1 activity was assessed by its ability to inhibit cell proliferation in the CCL64 cell line.

MATERIALS AND METHODS

Materials

We used the following bioabsorbable membranes: Paroguide (Vebas, Milano, Italy) made of 96–98% equine collagen (a mixture of 97% type I and 3% type III collagen), crosslinked with DPPA, and 2–4% chondroitin-4-sulfate GAG; Opocrin (Vebas, San Giuliano Milanese, Milano, Italy) made of type I (97%) and type III (3%) equine collagen crosslinked with DPPA; Biomed Extend made of bovine type I collagen crosslinked with GA (Sulzer Calciteck Inc., Carlsbad); Bio-Gide made of pork type I and III collagen not crosslinked (Geistlich Pharma AG, Wolhusen, Switzerland). All membranes have a thickness of 0.5 mm and a pore size of approximately 0.005 μ.

Cell cultures

The human dermal fibroblast cell line (BS PRC 41) was provided by Zooprofilattico (Brescia, Italy). Human fibroblasts were expanded in sterile polystyrene Falcon flasks obtained from Becton Dickinson and Company (Lincoln Park, NJ) using Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Gibco, Paisley, UK). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C.

Cultures on barrier membranes

The human dermal fibroblasts were seeded at a density of 1×10^6 cells/mL into wells containing bioabsorbable membranes (Ø 3 cm). After 24 or 48 h of plating (sub-confluent or confluent cultures) in MEM supplemented with 10% FCS, the membranes were transferred to new wells containing MEM in the absence of FCS and tested for ^{3}H-thymidine incorporation, collagen neosynthesis, GAG neosynthesis, and for TGFβ_1 activity as outlined below.

^{3}H-thimidine incorporation

Sub-confluent cultures of the human dermal fibroblast cell line were maintained for 24 h on Paroguide, Opocrin, Biomed Extend, and Bio-Gide membranes in MEM without serum containing 1 μCi/mL ^{3}H-thymidine (specific activity 13.4 Ci/mmol; Radiochemical Amersham, Little Chalfont, UK). At the end of the *in vitro* maintenance, the medium was discarded and the cells were washed with 0.1*M* phosphate buffer and solubilized in 0.5*M* NaOH. An aliquot of cell lysate was precipitated with 10% trichloroacetic acid (TCA) (30 min at 4°C), filtered into Millipore filters (Ø 0.45 μM; Millipore S.p.a., Milano, Italy), and washed with cold 5% TCA. The filters holding the acid-insoluble fraction were dried and counted in 10 mL of scintillation fluid (Filter Counter; Packard, Meriden, CT) in a Packard Tri-Carb 2125 liquid scintillation counter. Incorporation of radioactive into TCA insoluble fraction was expressed as counts per minute/culture. Total cell radioactivity was measured in separate aliquots of solubilized cells counted in 10 mL of scintillation fluid. Percent incorporation was calculated as the ratio between ^{3}H-thymidine incorporation into DNA (TCA-insoluble fraction) and total cell radioactivity.[2]

Isolation of newly neo-synthesized GAG

Confluent cultures of the human dermal fibroblast cell line were maintained for 24 h on Paroguide, Opocrin, Biomed Extend, and Bio-Gide membranes in the presence of MEM without serum containing 5 μCi/mL ^{3}H-glucosamine (specific activity 29 Ci/mmol; NEN Du Pont de Nemours, Boston, MA). At the end of the *in vitro* maintenance, the cells and media were collected separately. The cells were scraped into 1 mL of ice cold 0.1*M* Tris-HCl, 1.5 mmol/L $CaCl_2$ (pH 7.2), and lysed using a Bronson sonicator. The media were dialyzed, lyophilized, and dissolved in the above buffer. Both media and cell lysate were boiled for 5 min and then digested for 3 days at 37°C with 1 mg/mL of predigested (for 30 min at 37°C) pronase (Sigma Chemical Co., St. Louis, MO) in the presence of 1% (w/v) toluene. Fresh pronase was added daily. Proteins were removed through precipitation with 10% TCA (w/v) and centrifugation (12,000*g* for 30 min). The supernatants were collected and GAG was precipitated with 3 vols of 5% potassium acetate (w/v) in absolute ethanol (for 24 h at 4°C) and pelleted by centrifugation for 20 min at 12,000*g*. The pellets were dissolved in 0.1*M* Tris-HCl pH 7.2. Aliquots of labeled GAG were mixed with 10 mL of scintillation fluid Ultima Gold (Packard) and counted in a Packard Tri-Carb 2425 liquid scintillation counter.[10] The radioactivity was described as extracellular radioactivity (recovered in the medium and bound to the membranes) and cellular radioactivity (recovered in the intra- and pericellular compartment). Radioactivity was expressed as counts per minute/milligram cell protein.

Analysis of secreted collagen

Confluent cultures of the human dermal fibroblast cell line were maintained for 24 h on Paroguide, Opocrin, Biomed Extend, and Bio-Gide membranes in the presence of MEM without serum supplemented with L-ascorbic acid (50 μg/mL), β-aminopropionitrile fumarate (50 μg/mL), and 5 μCi/mL ^{3}H-proline (s.a. 35 Ci/mmol; NEN Du Pont de

TABLE I
DNA Synthesis in the Human Dermal Fibroblast Cell Line Cultured for 24 h in MEM Plus ^{3}H-Thymidine on Biomembranes

	Counts per Minute/Culture		
	Total Cell Radioactivity	TCA Insoluble Fraction	% Incorporation
Paroguide	68,705 ± 5340	1733 ± 82*	2.5
Opocrin	63,052 ± 3155	804 ± 54**	1.3
Biomed Extend	42,355 ± 1076**	373 ± 30*,**	0.9
Bio-Gide	40,282 ± 2521**	218 ± 31*,**	0.5

*Differences versus Opocrin membrane: F test significant at 95%.
**Differences versus Paroguide membrane: F test significant at 99%.

Nemours). At the end of the *in vitro* maintenance, the cells and media were collected separately. Collagen was extracted according to Webster and Harvey.[11] Briefly, cell layers were treated with 100 μL of cold acetic acid (0.5*M*) containing 1 mg/mL pepsin. The culture media were dialyzed against 1% acetic acid, lyophilized, and then redissolved in the same buffer of the cell.

Rat skin collagen (200 μg/mL) was used as a carrier and the samples were centrifuged at 4000*g* for 20 min. Collagen was precipitated from the supernatants by the addition of 250 μL of NaCl in 0.5*M* acetic acid (25% w/v).The precipitates were redissolved in 300 μL of 0.15*M* NaCl in 0.05*M* Tris-HCl pH 7.5 and centrifuged. The final precipitates were dissolved in 250 μL of acetic acid (0.5*M*) and transferred to vial inserts containing 2 mL of scintillation liquid. Radioactivity was expressed as counts per minute/milligram protein. The specificity of the assay was determined by susceptibility of the precipitated collagen to purified bacterial collagenase.

Preparation of conditioned media (CM)

Confluent cultures of the human dermal fibroblast cell line grown on Paroguide, Opocrin, Biomed Extend, and Bio-Gide membranes were washed with saline solution and cultured for 12 h in serum-free medium MEM.[12] This medium was discarded to avoid contamination by seric factors. The cells were cultured for the next 24 h in MEM alone. CM were collected and centrifuged for 10 min at 350*g* to remove cell debris, then dialyzed, lyophilized, and used for TGFβ$_1$ bioassay.

TGFβ$_1$ bioassay

The bioassay was done as described by Meager.[13] Briefly, CM was dissolved in MEM containing 10% FCS and 1% nonessential amino acids and TGFβ$_1$ activity was determined by measuring the inhibition of ^{3}H-thymidine incorporation (s.a. 6.7 Ci/mmol, NEN) by Mv-1-Lu mink lung epithelial cells (ATCC/CCL-64). CCL-64 cells were seeded in 96-well microtiter plates at low cell density (10,000 cell/well) in the growth medium containing TGFβ$_1$ (0.5 ng/mL) or CM at the protein concentration reported in the Tables. The specificity of TGFβ$_1$ activity was determined by the addition of anti-TGFβ$_1$ antibody. The inhibition of ^{3}H-thymidine incorporation neutralized by the antibody addition was attributed to TGFβ$_1$. All cultures were maintained for 72 h and pulsed with 2.5 μCi/mL ^{3}H-thymidine for the last 6-h incubation. Cells were harvested with a semiautomatic cell harvester and the amount of ^{3}H-thymidine incorporated was determined by the standard liquid scintillation method.

Protein determination

Protein concentrations were determined by Lowry assay[14] on aliquots of cell lysate.

Statistical analysis

Results reported in the tables are the mean ± SD (standard deviation) of three separate experiments, each performed in quadruplicate. Statistical analysis was performed by Student's two-tailed *t* test and by analysis of variance (ANOVA) followed by Scheffé F test.

RESULTS

Cell growth

The human dermal fibroblast cell line was cultured for 24 h in MEM on collagen membranes in the absence of FCS to avoid seric contamination. The results showed that the proliferative capacity varied with the culture condition (Table I). Radioactivity incorporation into DNA (TCA insoluble fraction) was highest with the Paroguide membrane. The lowest level of radioactivity incorporated into the DNA occurred with the Bio-Gide membranes, followed by Biomed Extend. Radioactivity incorporation into DNA with the Opocrin membrane that, like Paroguide, is crosslinked with DPPA but without chondroitin-4-sulfate, was significantly less than with Paroguide, but significantly more than with Biomed Extend and Bio-Gide.

The percent of ^{3}H-thymidine incorporation, as a ratio of incorporation into TCA insoluble fraction to

TABLE II
GAG Neosynthesis in the Human Dermal Fibroblast Cell Line Cultured for 24 h in MEM Plus ^{3}H-Glucosamine on Biomembranes (Counts per Minute/Milligram Protein)

	Cellular GAG	Extracellular GAG		Total Extracellular GAG
		Media	Biomembranes	
Paroguide	18,751 ± 534	13,926 ± 207	74,019 ± 3342*	87,945
Opocrin	10,476 ± 1020	20,032 ± 843	41,596 ± 1607**	61,628
Biomed Extend	5158 ± 344	12,866 ± 1116	24,087 ± 1003*,**	36,953
Bio-Gide	6876 ± 820	7888 ± 329	25,345 ± 815*,**	33,233

*Differences versus Opocrin membrane: F test significant at 99%.
**Differences versus Paroguide membrane: F test significant at 99%.

total cell radioactivity, was approximately 0.5% in human fibroblasts cultured on Bio-Gide and increased to 0.9% with Biomed Extend, 1.3% with Opocrin and 2.5% with Paroguide.

GAG neosynthesis

The human dermal fibroblast cell line synthesized and released into the medium large quantities of GAG and extracellular GAG was more than cellular GAG with all the substrates (Table II). The cellular GAG accumulation in the human fibroblast cell line grown on Biomed Extend and Bio-Gide showed no significant differences, whereas it increased approximately 2-fold with Opocrin and 3.6-fold with Paroguide.

Cells cultured on the membranes released an aliquot of extracellular labeled GAG into the medium; the rest was bound to the membranes, forming a complex that was not easily dissociable. With Bio-Gide, Biomed Extend, and Paroguide membranes, extracellular GAG accumulation in the media was less than with Opocrin. Between 65 and 84% of extracellular GAG was bound to the membranes. Paroguide membrane bound the most extracellular GAG, Biomed Extend and Bio-Gide the least. When the total level of extracellular GAG (in the medium and bound to the membranes) was considered, ^{3}H-glucosamine incorporation by Opocrin, Biomed Extend, and Bio-Gide was 30, 58, and 62% less, respectively, than by Paroguide.

Collagen neosynthesis

Fibroblasts were cultured for 24 h (Table III) in MEM without serum on Paroguide, Opocrin, Biomed Extend, and Bio-Gide membranes in the presence of ^{3}H-proline. Biomed Extend and Bio-Gide membranes accumulated the least collagen proteins in the cellular pool. Opocrin membrane increased them 2.1-fold, and Paroguide membrane 4.5-fold compared with Biomed Extend. In the extracellular compartment, collagen proteins were present in the medium and bound to the membranes. The total amount of collagen accumulated in the extracellular environment with Paroguide was significantly greater than with Opocrin (1.6-fold), Biomed Extend (2.9-fold), and Bio-Gide (3.1-fold).

TGFβ_1 activity

The activity of secreted TGFβ_1 in the CM by the human dermal fibroblast cell line cultured on collagen membranes was assayed by its inhibition of ^{3}H-thymidine incorporation in CCL 64 cell line (Table IV). TGFβ_1 (0.2 ng/mL) inhibited the proliferative response of CCL 64 by approximately 95%. CM from the human fibroblast cell line inhibited cell proliferation in a dose-dependent manner. The TGFβ_1 activity, dosed in the CM of cells cultured on Paroguide and Opocrin membranes, was higher than in the CM of cells cultured on Biomed Extend and Bio-Gide. Adding the

TABLE III
Collagen Neosynthesis in the Human Dermal Fibroblast Cell Line Cultured for 24 h in MEM Plus ^{3}H-Proline on Biomembranes (Counts per Minute/Milligram Protein)

	Cellular Collagen	Extracellular Collagen		Total Extracellular Collagen
		Media	Biomembranes	
Paroguide	318 ± 22	146 ± 15	31,323 ± 1290*	31,469
Opocrin	154 ± 9	138 ± 9	19,781 ± 1316**	19,919
Biomed Extend	71 ± 11	98 ± 12	10,888 ± 522*,**	10,986
Bio-Gide	83 ± 11	54 ± 10	10,034 ± 209*,**	10,088

*Differences versus Opocrin membrane: F test significant at 99%.
**Differences versus Paroguide membrane: F test significant at 99%.

TABLE IV
Percentage of Inhibition of ^{3}H-Thymidine Incorporation Into the CCL-64 Cell Line in the Presence of CM Obtained From Human Dermal Fibroblast Cell Line Cultured for 24 h on Biomembranes

	Protein Concentration	% Inhibition Attributed to TGFβ$_1$	% Inhibition by Anti-TGFβ$_1$
TGFβ$_1$	0.5ng/mL	95	4
Paroguide	8μg/mL	91	5
	4μg/mL	77	5
	2μg/mL	45	—
Opocrin	8μg/mL	90	3
	4μg/mL	72	4
	2μg/mL	43	3
Biomed Extend	8μg/mL	65	5
	4μg/mL	46	2
	2μg/mL	29	4
Bio-Gide	8μg/mL	68	4
	4μg/mL	39	4
	2μg/mL	20	3

Percentage of inhibition was compared with untreated CCL-64 cell line. The values represent the mean of four plates. For all determinations, standard deviation was <6%.

anti-TGFβ$_1$ antibody to the CM neutralized TGFβ$_1$ activity, thereby indicating the growth inhibition was attributed to the growth factor.

DISCUSSION

Before use in reconstructive surgery, collagen-derived biomaterials require a chemical stabilization process to improve their biochemical and mechanical properties. In fact, when not crosslinked, collagen membranes contract because of their low thermal stability, partial denaturation, and dissolution of biomembranes after incubation at 37°C, and this leads to low cell proliferation.[15] Chemical agents, capable of inducing crosslinking to increase resistance to collagenase-induced degradation and to decrease immunogenicity, alter the surface chemistry of the biomaterials and can directly affect cell attachment, cell growth, and ECM and growth factor production. In this study, we determined which collagen membrane was the most biocompatible: Paroguide which is crosslinked with DPPA and contains chondroitin sulfate; Opocrin which is crosslinked with DPPA; Biomed Extend which is crosslinked with GA; and Bio-Gide which is left untreated.

Paroguide membrane, which contains chondroitin sulfate, increased cell proliferation more than Opocrin, Biomed Extend, and Bio-Gide. Our data concur with those of Emonard et al.[16] who reported that when fibroblasts are cultured in Matrigel, composed of laminin, collagen, and heparan sulfate proteoglycan, they reach confluence more quickly than cells grown on other substrates. Matrigel, like Paroguide, could promote cell growth through enhanced matrix deposition. In fact, the experiments investigating collagen and GAG accumulation showed Paroguide membrane enhanced GAG and collagen secretion much more than others. The Opocrin membrane, which was crosslinked with DPPA like Paroguide membrane, but which did not contain chondroitin sulfate, showed less GAG and collagen accumulation than Paroguide, but more than Biomed Extend and Bio-Gide.

In our experimental conditions, the majority of secreted matrix macromolecules interacted with the membranes themselves, thus probably contributing to the formation of a substrate that was particularly suitable for cell proliferation and for the laying down of new ECM macromolecules. The considerably greater amount of organic macromolecules in the extracellular environment, observed mainly with Paroguide and Opocrin, may encourage a greater concentration of growth factors and cytokines which, in turn, facilitate tissue regeneration.

The nature of the *in vitro* substrate influences cell behavior and ECM composition which, in turn, modulates tissue differentiation and regeneration.[2,17–20] The distribution and organization of GAG and collagen fibers varies with tissue type, and stage of tissue regeneration.[21] This diversity of composition, organization, and distribution arises not only from the differential expression of genes for the various molecules in specific tissues, but also from the ability of the matrix macromolecules to bind to a variety of substances including carbohydrates, proteins, and proteoglycans. Any one of these substances may be important for cell proliferation and tissue regeneration through either transmembrane signal or cell–substrate interaction.[22] The ECM can also exert its effects indirectly on cells by binding and interacting with growth factors and cytokines.[23] ECM can protect these molecules from being degraded or can present them more efficiently to their receptors. Growth factors and cytokines, in turn, induce the cells to modify the production of the ECM.[24,25]

The healing of a dermal wound and tissue regeneration require a coordinated sequence of biologic events beginning with platelet-induced homeostasis, followed by an influx of inflammatory cells and fibroblasts, the formation of new ECM and blood vessels (granulation tissue), and the proliferation of cells to reconstitute the tissue. TGFβ$_1$ has an important part in each of these events, which can largely be reproduced in normal tissue with the administration of the growth factor.[26]

In the light of these data, we investigated TGFβ$_1$ production by human fibroblasts cultured on DPPA- and GA-treated membranes. Fibroblasts cultured on DPPA-crosslinked membranes produced more TGFβ$_1$ than fibroblasts cultured on the GA-crosslinked Biomed Extend and on the not-crosslinked Bio-Gide

membranes, with the Paroguide membrane increasing $TGF\beta_1$ secretion more than Opocrin.

These results confirm those of previous cytotoxic studies using fibroblasts, human dermis, or endothelial cells cultured on biomaterials crosslinked with GA or DPPA.[27,28] DPPA treatment allows more rapid, extensive development of endothelial-like cells and provides better support than GA treatment for fibroblast growth and dermal formation.[15,28]

It is not clear why not-crosslinked Bio-Gide membranes are the least suitable for cell proliferation and ECM deposition. Because they show the same mechanical properties as the other membranes, one can speculate they have been submitted to some stabilizing chemical or physical procedure other than crosslinking that increased their cytotoxicity.

Our results confirm that the DPPA method is preferable to the GA method and that membranes made of collagen and chondroitin sulfate are more biocompatible than membranes made of pure collagen.

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References

1. McGinnis M, Larsen P, Miloro M, Beck FM. Comparison of resorbable and nonresorbable guided bone regeneration materials: A preliminary study. Int J Oral Maxillofac Implants 1998; 13:30–35.
2. Locci P, Calvitti M, Belcastro S. Phenotype expression of gingival fibroblasts cultured on membranes used in guided tissue regeneration. J Periodontol 1997;68:857–863.
3. Jorge-Herrero E, Fernandez P, Turnay J. Influence of different chemical crosslinking treatments on the properties of bovine pericardium and collagen. Biomaterials 1999;20:539–545.
4. Huang-Lee LLH, Cheung DT, Nimni M. Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks. J Biomed Mater Res 1990; 24:1185–1201.
5. Van Luyn MJA, Van Wachen PB, Olde Damink LHH, Dijkstra PJ, Feijen J, Nieuwenhuis P. Secondary cytotoxicity of crosslinked dermal sheep collagens during repeated exposure to human fibroblasts. Biomaterials 1992;13:1017–1024.
6. Gough JE, Scotchford CA, Downes S. Cytotoxicity of glutaraldehyde crosslinked collagen/poly(vinyl alcohol) films is by the mechanism of apoptosis. J Biomed Mater Res 2002;61(1):121–130.
7. Schmidt CE, Baier JM. Acellular vascular tissue: Natural biomaterials for tissue repair and tissue engineering. Biomaterials 2000;21:2215–2231.
8. Petite H, Frei V, Huc A, Herbage D. Use of diphenylphosphoryl azide for crosslinking collagen-based biomaterials. J Biomed Mater Res 1994;28:150–165.
9. Roche S, Ronzière MC, Herbage D, Freyria AM. Native and DPPA crosslinked collagen sponges seeded with fetal bovine epiphyseal chondrocytes used for cartilage tissue engineering. Biomaterials 2001;22:9–18.
10. Locci P, Lilli C, Marinucci L, Baroni T, Pezzetti F, Becchetti E. Embryonic skin fibroblasts release TGFα and TGFβ able to influence synthesis and secretion of GAG. Cell Mol Biol 1993; 39:415–426.
11. Webster DF, Harvey W. A quantitative assay for collagen synthesis in microwell fibroblast cultures. Anal Biochem 1979; 96:220–224.
12. Lilli C, Marinucci L, Bellocchio S, Ribatti D, Balducci C, Baroni T, Cagini L, Giustozzi G, Locci P. Effects of transforming growth factor-β1 and tumour necrosis factor-α on cultured fibroblasts from skin fibroma as modulated by toremifene. Int J Cancer 2002;98:824–832.
13. Meager A. Assays for transforming growth factor β. J Immunol Methods 1991;141:1–14.
14. Lowry OH, Rosebrough NJ, Farr AJ, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951;193:265–275.
15. Vaissiere G, Chevallay B, Herbage D, Damour O. Comparative analysis of different collagen-based biomaterials as scaffolds for long-term culture of human fibroblasts. Med Biol Eng Comput 2000;38:205–210.
16. Emonard H, Grimaud JA, Nusgens B, Lapière ChM, Foidart JM. Reconstituted basement-membrane matrix modulates fibroblast activities *in vitro*. J Cell Physiol 1987;133:95–102.
17. Evangelisti R, Stabellini G, Becchetti E, Carinci P. Exogenous glycosaminoglycans modulate extracellular and intracellular accumulation of newly synthesised glycosaminoglycans by embryonic chick skin fibroblasts. Cell Biol Int Rep 1989;13:437–446.
18. Locci P, Becchetti E, Pugliese M. Metal substrates influence the release of GAG and TGFβ by human bone cells. J Periodontol 1996;67:1260–1266.
19. Marinucci L, Lilli C, Baroni T. *In vitro* comparison of bioabsorbable and non-resorbable membranes in bone regeneration. J Periodontol 2001;72:753–759.
20. Stabellini G, Locci P, Calvitti M, Evangelisti R, Marinucci L, Bodo M, Caruso A, Canaider S, Carinci P. Epithelial-mesenchymal interactions and lung branching morphogenesis. Role of polyamines and transforming growth factor β_1. Eur J Histochem 2001;45:151–162.
21. Adams JC, Watt FM. Regulation of development and differentiation by the extracellular matrix. Development 1993;117: 1183–1198.
22. Martins-Green M, Bissell MJ. Cell-ECM interactions in development. Dev Biol 1995;6:149–159.
23. Locci P, Marinucci L, Lilli C, Martinese D, Becchetti E. Transforming growth factor β_1-hyaluronic acid interaction. Cell Tissue Res 1995;281:317–324.
24. Bodo M, Becchetti E, Giammarioli M. Interleukin-1 and interleukin-6 differentially regulate the accumulation of newly synthesised extracellular matrix components and the cytokine release by developing chick embryo skin fibroblasts. Int J Dev Biol 1994;38:535–542.
25. Locci P, Baroni T, Lilli C. TGFβ and TGFα antagonistic effect *in vitro* on extracellular matrix accumulation by chick skin fibroblasts at two distinct embryonic stages. Int J Dev Biol 1999;43: 157–165.
26. Sporn MB, Roberts AB. Transforming growth factor β: Recent progress and new challenges. J Cell Biol 1992;119:1017–1021.
27. Petite H, Duval JL, Frei V, Abdul-Malak N, Sigot-Luizard MF, Herbage D. Cytocompatibility of calf pericardium treated by glutaraldehyde and by the acyl azide methods in an organotypic culture model. Biomaterials 1995;16:1003–1008.
28. Imamura E, Sawatani O, Koyanagi H, Noishiki Y, Miyata T. Anticalcification effects of epoxy compounds as crosslinking agent. Jpn J Artif Organs 1988;17:1101–1103.