

Light and electron microscopic evaluation of biocompatibility, resorption and penetration characteristics of human collagen graft material

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Abstract. This study was initiated to test the biocompatibility, resorption and penetration characteristics of human collagen graft material in vitro and in vivo using light (LM) and electron microscopy (EM). To study this relationship, pieces of glutaraldehyde cross-linked collagen sponges ($1 \times 1 \times 0.5$ cm), were: (1) cultured in sterile Petri dishes with human gingival fibroblasts and human periodontal ligament fibroblasts for 2 weeks; (2) implanted in subcutaneous pockets made in both thighs (total 20 sites) of 10 Sprague-Dawley rats for 7–56 days. The behaviour of the growth of the fibroblasts was studied by inverted light microscopy (LM), then tissue culture specimens were studied from without and within using low-temperature scanning electron microscopy (LTSEM). Blocks obtained from the graft sites of the rat were processed for LM and transmission EM. Long-term LM observations showed attachment and random orientation of cells on and around the collagen sponge in culture during the first 48 h. Between 7 and 14 days, the majority of the cells adjacent to the sponge were orientated at right angles to its margin with their long axes approximately parallel to each other. The LTSEM revealed that large numbers of HGF and HPLF grew onto the collagen sponges, but no cellular penetration to the middle of the sponge was seen. LM and TEM of the rat specimens showed a cellular reaction to the collagen graft, as well as slow resorption, and fibroblast invasion of the graft at 6–8 weeks. It was concluded that the human collagen graft was biocompatible with HGF and HPLF, with penetration first observed at 42 days post-implantation. In the in vivo study, the collagen underwent slow resorption over a period of 8 weeks.

Key words: collagen graft; fibroblast; light and electron microscopy.

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To date, there is considerable interest in the use of collagen graft material in reparative surgical procedures. Several authors have evaluated the use of reconstituted collagen membranes in periodontal bony defects of *experimental animals* and showed that such membranes have the capacity of connective tissue regeneration and partial prevention of epithelial cell migration, onto the root surface (Pitaru et al. 1987, 1988, Blumenthal 1988). Furthermore, the use of enriched collagen solution and combined collagen-decalcified bone in periodontal defects in dogs demonstrated that collagen material promoted wound healing and new attachment for-

mation and reinforced the osteoinductive activity (Yaffe et al. 1984, Blumenthal et al. 1986). Furthermore, hemostatic properties and promotion of wound healing provide this biomaterial with considerable potential in a wide range of clinical applications. However, when collagen material is treated with glutaraldehyde, the physicochemical nature of the graft surface, the pore sizes and the interconnections between the pores are changed.

Thus, the aim of this study was to test the growth, penetration and orientation of human gingival and periodontal fibroblasts onto the collagen graft in tissue culture, as well as to test

the resorption and penetration pattern in rats, using both light and electron microscopy. In practical terms, the collagen graft for use in periodontal surgical procedures should exhibit fibroblast compatibility, which can be demonstrated by active colonization by these cells, with defined penetrability and resorption characteristics.

Material and Methods

Derivation of fibroblasts

Premolars extracted for orthodontic reasons were obtained from children or young adults in the Oral Surgery department of the Dental Hospital, Card-

iff. Usually, 1 or 2 teeth were obtained from 1 patient for each procedure and washed in sterile phosphate buffered saline (PBS) 3 times. The gingiva attached to the coronal 1/3 of the root was carefully dissected away to circumvent contamination by gingival fibroblasts. The crowns of the teeth up to the gingival tissue were dipped in 1% sodium hypochlorite solution for 5 min. The teeth were then rinsed in 3 changes of PBS, placed in a 10 ml sterile bottle with 5 ml of 0.125% trypsin and incubated on a rotary mixer for 5 min at 37°C. After incubation, the tooth was washed with minimal essential medium (MEM) supplemented by 10% foetal calf serum (FCS). The mid 1/3 of the root, moistened with MEM, was scraped in such a way that the periodontal ligament would fall into a Petri dish containing a few drops of the MEM. Tissue removed in this way was divided until approximately 1 mm³ in size. A sterile cover slip (22 × 32 mm) was placed over the tissue fragments which were cultured in 5 ml of MEM with 10% FCS in an air/CO₂ 95%/5% atmosphere at 37°C.

The gingival tissue was obtained from patients undergoing reverse bevel flap surgery and cultured in the same way as the periodontal ligament specimens. After outgrowth of the cells from the explants in both situations, the coverslip was removed, cells were released by trypsinization and grown in 75 cm² culture flasks. Medium, which was changed twice weekly until cultures reached confluence, contained 10 ml of Dulbecco's modified Eagle's solution × 10.2 ml Hepes buffer, pH 7.4, 2 ml of L-glutamine and 2 ml of penicillin-streptomycin added to 80 ml of distilled water and adjusted to pH 7.4. 10% FCS was added to the medium prior to use.

Extraction of collagen

Human collagen Type I was extracted from human placenta using a modification of the Chung & Miller (1974) procedure and characterised by sodium dodecyl sulphate electrophoresis using a standard sample of human Type I collagen.

Preparation and crosslinking of collagen sponges

Collagen sponge was prepared by making a 1% (w/v) dispersion of Type I

human collagen in 0.01 M HCl pH 2, using a mechanical mixer for homogenization. The dispersion was deaerated for 5 minutes at room temperature and 30 ml poured into a 93 × 73 × 60 mm glass container and spread evenly before freezing for 2 hours at -25°C. The container and the frozen dispersion were transferred to another freezer (-60°C) and left for a few hours before freeze-drying at -60°C. After freeze-drying, the collagen sponge was crosslinked by immersion in 50 ml of 0.25% (v/v) glutaraldehyde solution* pH 4.5–5.5, for 48 h at room temperature. After washing with 80 ml of distilled water 3 times daily for one week at room temperature, the collagen sponge was frozen again at -60° and then freeze-dried as before.

The morphology of the freeze-dried and crosslinked sponge was investigated by scanning and transmission electron microscopy (SEM/TEM). Samples of freeze-dried crosslinked collagen sponge were fixed on aluminium stubs, sputter coated with gold in an EM scope sputter apparatus**. These samples were observed in a JEOL 846 A SEM at 1–20 kvs. Also, TEM was carried out as in the in vivo experiment described below.

In vitro experiment

A piece of collagen sponge (10 × 10 × 2 mm) was held to the bottom of a Petri dish by using two pieces of glass microscope slide (2 × 4 cm) applied to the edges of the sponge.

Either periodontal ligament or gingival fibroblasts which had reached confluence were washed 3 × with PBS and 5 ml of 0.25% trypsin was added for 30 s. After removal of the trypsin solution, cells were allowed to round up before addition of 5 ml of MEM with 10% FCS. Fibroblasts were counted in a haemocytometer and the suspension adjusted to 1 × 10⁵ cells in 5 ml of MEM. 5 ml of this cell suspension was added to the Petri dish with the collagen sponge and incubated at 37°C. The behaviour of the growth of the fibroblasts was studied by inverted light microscopy for two weeks. After 1 week, the medium was removed, the collagen sponge studied from both without and within (using

vertical sectioning of the sponge) by low temperature scanning electron-microscopy using an EMscope SP 2000 A cryo system. In this procedure, the collagen sponge was mounted on special low temperature stubs and then rapidly frozen in nitrogen slush (below -130°C). The specimen was etched in the SEM for 30 s at -95°C to remove variable amounts of surface frost by sublimation. It was then sputter coated with gold at -130°C in the SP 2000 A and then observed and photographed on the low temperature controlled stage in a JEOL JSM – 840A scanning electron microscope.

In vivo experiment

In order to evaluate the cellular reaction and ingrowth into the collagen grafts in vivo, these were implanted into 10 Sprague-Dawley rats (female, 10 weeks old, approximately 250 g weight). The animals were anaesthetized using ether and the hair on the flank area of both legs was shaved before the collagen grafts (4 × 4 × 2 mm) were implanted subcutaneously in small pockets prepared at these sites. 2 animals were sacrificed at 1, 2, 4, 6 and 8 weeks post-implantation yielding 4 specimens in each 2 animal group. A sample containing the recipient site was dissected out and divided into approximately equal portions. One portion was fixed in 10% formal saline for 48 h and then embedded in paraffin wax. Serial sections were cut through these paraffin wax embedded specimens and stained with haematoxylin and eosin (H & E). 4 slides, selected at random, one from each specimen, were used to quantify the cellular response to the collagen implant in these animals, as follows. The various cells (i.e., neutrophils, mononuclear cells, macrophages, plasma cells, multi-nucleated (MN) giant cells and fibroblasts) in 1 mm² were counted at three areas of both the edge and centre of each slide, using a light microscope (× 400) with an eye piece graticule. The median values of the 3 countings at each of these areas were calculated and used to compute the means and standard deviation values of the 4 slides, which were taken as the cell count for the site. The other portion was cut into small pieces (approximately 1 mm³) and immediately fixed in neutral buffered 2.5% glutaraldehyde for 3 h. These pieces were washed in buffer overnight and post-fixed for 3 h in neutral buffered 2%

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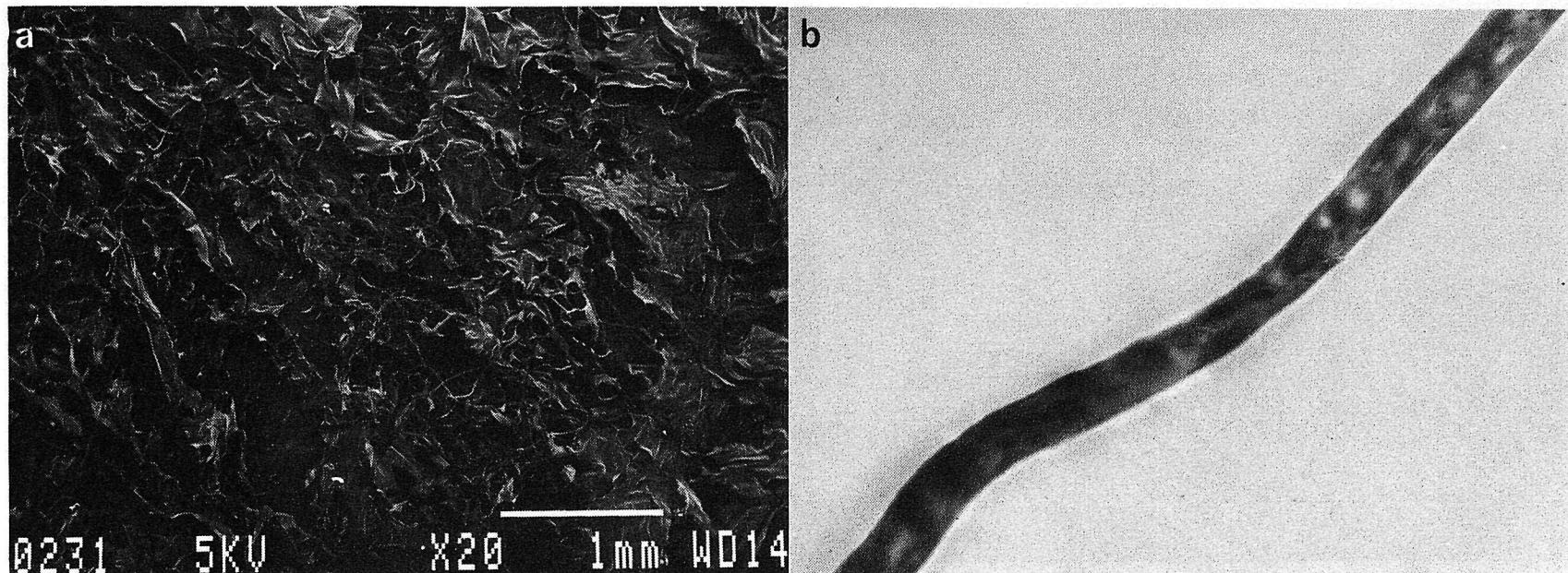


Fig. 1. (a) SEM view of the crosslinked collagen sponge on its outer surface (original magnification $\times 20$). (b) TEM view of the crosslinked collagen sponge in vertical section (original magnification $\times 4200$).

osmium tetroxide. The tissue pieces were dehydrated in an ethanol gradient (70, 90 and 100%), 3 \times 20 min each and then cleared in trichloroethane* 2 times for 15 min each on a rotary device. The pieces were infiltrated with Araldite CY 212 overnight, then embedded in Araldite in BEEM type capsules and polymerised at 65°C for 48 h. Thin sections of 90 nm or less were cut, viewed and photographed in a Philips EM 400 TEM.

Results

The morphology of the crosslinked collagen sponge on its outer surface and on the cut surface is shown in Fig. 1. The cross-linked collagen sponge is composed of an interlaced, densely packed collagen network. This confers on the surface of the sponge an uneven sheet-like structure with pores of various sizes with closed-end channels, as shown by SEM (Fig. 1a). Examination by TEM (Fig. 1b) revealed that the collagen fibrils of the crosslinked collagen sponge had a rope-like appearance.

In vitro growth of fibroblasts on sponge

In the in vitro experiments, long-term light microscope observations and the collagen sponge in cultures of human gingival (HGF) and periodontal ligament fibroblasts (HPLF) showed no obvious differences between these two cell lines with regard to cell attachment, mi-

gration and orientation. Therefore the events observed in tissue culture from both cell lines, using light microscopy are reported.

At 12 to 48 h of culture, HGF and HPLF were attached to the culture dish within the first 12 h after seeding. These cells appeared to be randomly orientated on and around the collagen sponge in culture, with their normal appearance, i.e., elongated spindle-shaped, bipolar and with a flat surface morphology.

From 48 h through to 6 days in culture, the cells increased in number with an apparent migration towards the graft; there was little orientation which varied from 0–90°.

The cells reached confluence during the period of seven to 14 days in culture. The majority of the cells adjacent to the sponge were directionally orientated at right angles to its margin with their long axis approximately parallel to each other (Fig. 2a, b).

Examination of the cultures by low-temperature SEM showed that the growth of human gingival and periodontal ligament fibroblasts in the presence of the collagen sponge at 4 days resulted in adherence of these cells to the surface of the sponge. These low-temperature studies revealed that large numbers of both HGF and PDLF grew onto the sponge with normal spindle shaped morphology and long branching processes (Fig. 3). Using the same low temperature SEM techniques for the collagen sponge, cut in vertical sections, it was apparent that there had been no cellular penetration to the middle of the sponge after 1 week in culture Fig. 4a.

In vivo cellular reaction to the implanted sponges

Dissection of the 20 thigh sites of the rats at 1, 2, 4, 6 and 8 weeks yielded 4 samples at each time point. During dissection, it was apparent that the graft had become encapsulated. The histological evaluation of the tissue response to the collagen implant at 7 days post-implantation showed a marked cellular reaction, characterized by the presence of mononuclear cells, neutrophils and macrophages (Fig. 4 b, c). At 14 days post-implantation, the cellular reaction was still dominated by mononuclear cells, with the presence of macrophages, plasma cells and multinucleated (MN) giant cells. The cellular reaction seen at this stage by light microscopy was supported by TEM micrographs (Fig. 5a). At 28 days post-implantation, the chronic foreign body reaction persisted with less mononuclear cells and macrophages, but more plasma and MN giant cells. The cellular reaction seen at this stage by light microscopy was supported by TEM micrographs (Fig. 5a). At 28 days post-implantation, the chronic foreign body reaction persisted with less mononuclear cells and macrophages, but more plasma and MN giant cells, as compared to that of the 14 days reaction. No fibroblast ingrowth was seen at this stage. At 42 days, the cellular reaction was predominantly MN giant cells; with a smaller number of other chronic inflammatory cells. This was accompanied by fibroblast ingrowth into the collagen implant. This latter finding is also shown in the TEM micrograph (Fig. 5b). At 56 days post-implantation, a minimal reaction with a smaller number of inflammatory cells was seen in the histological preparations. At the same time, a higher number of fibroblasts appeared in the implant (Fig. 4 b, c). The overall inflammatory cellular reaction which was

*Inhibisol – Penetone, Cramlington, Northumberland, UK.

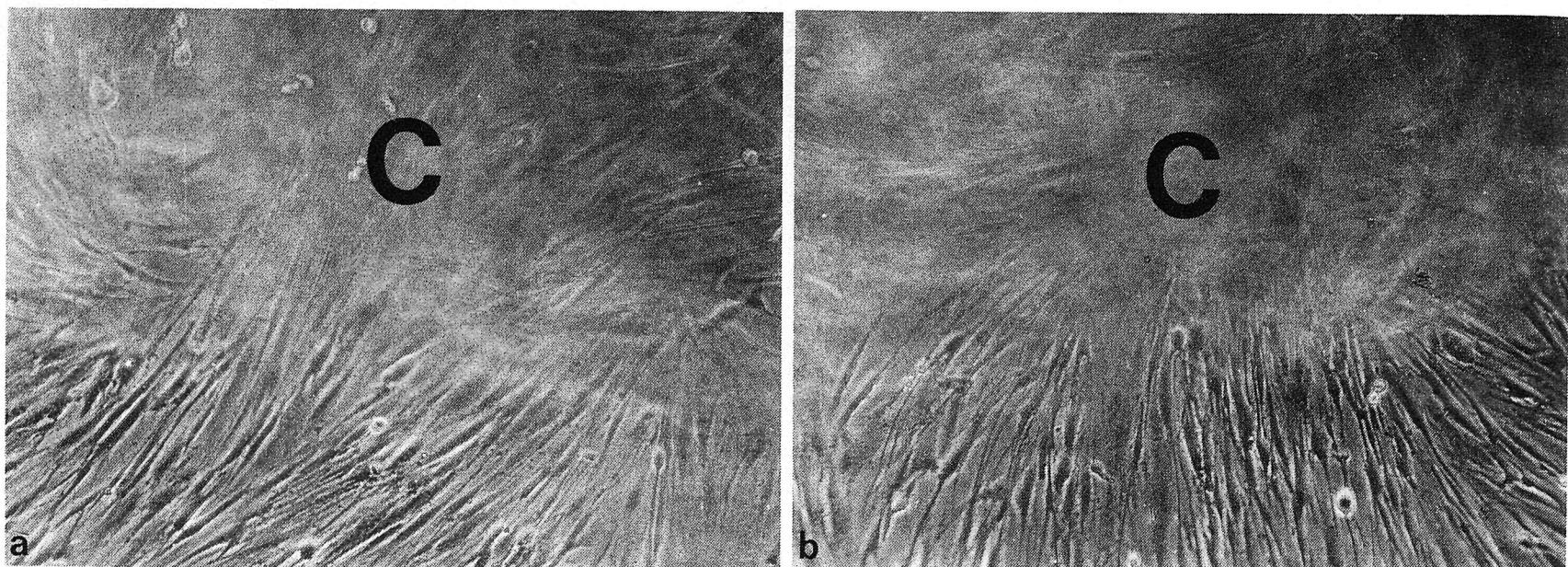


Fig. 2. (a) Human gingival fibroblasts (HGF) orientated to the margin of the collagen sponge (C) after 7 days in culture (original magnification $\times 40$). (b) Human periodontal ligament fibroblasts (HPLF) orientated to the margin of the collagen sponge (C) after 7 days in culture (original magnification $\times 40$).

apparent at the edge of the implant (Fig. 4b) was minimal within the implant itself (Fig. 4c). The collagen graft underwent slow resorption over the period of implantation.

Discussion

The use of reconstituted collagen membranes in periodontal bony defects of *experimental animals* demonstrated that these membranes have the capacity to support periodontal regeneration and to prevent, partially, the colonization of experimentally exposed root surfaces by epithelial cells during the initial stages of periodontal wound healing (Pitaru et al. 1987, 1988, 1989, Blumenthal 1988). In this study, human Type I collagen

graft was prepared with a view to its use in such surgical procedures. Therefore, the biocompatibility, penetration and resorption characteristics of the collagen graft in tissue culture and the animal model were evaluated using light and electron microscopy. The evidence presented in this study confirms that such evaluation procedures are a sensitive and precise test system for the biocompatibility of implantable collagen graft material. The in vitro experiments at day 5 and 7, post seeding, showed that HGF and PDLF were successfully grown upon the surface of human collagen graft material. The low-temperature scanning electron microscopy has shown that the cells were well preserved with a normal morphology in hydrated

preparations. Although these procedures have revealed a satisfactory growth of cells with superimposition, adhesion and orientation in relation to the graft, no penetration was seen over one week in culture. In practical terms, the evaluated collagen graft has met the requirement of compatibility *in vitro*, which can be demonstrated by active colonization and directional orientation by these cells, with defined penetrability. Many authors have reported that when fibroblasts are cultured in reconstituted collagen gels, the cells tend to orientate in a particular pattern and to reorganize their surrounding matrix by a similar process of cellular attachment and polarization (Bell et al. 1979, Bellows et al. 1981, Harris et al. 1981, Sto-

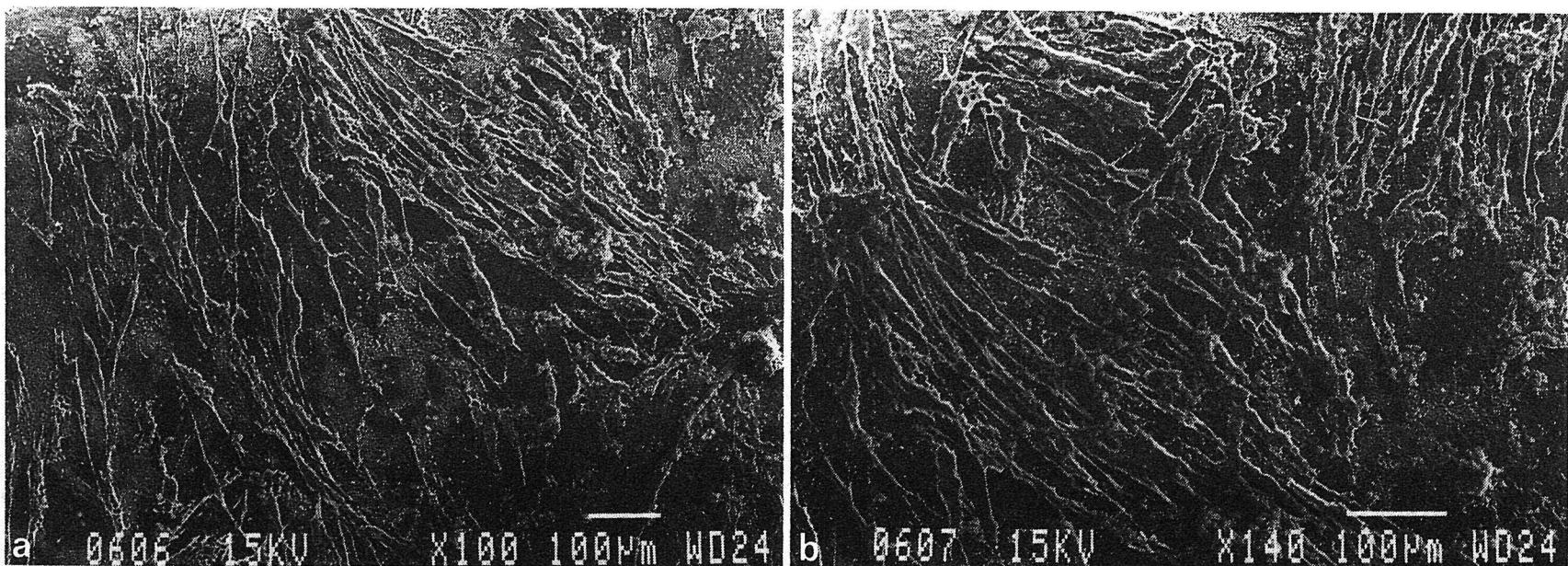


Fig. 3. (a) Low temperature SEM of human gingival fibroblasts (HGF) growing on the surface of collagen sponge at 5 days in culture (original magnification $\times 100$). (b) Low temperature SEM of human periodontal ligament fibroblasts (HPLF) growing on the surface of collagen sponge at 5 days in culture (original magnification $\times 100$).

pak et al. 1982). This in vitro interaction of fibroblasts with the extracellular matrix components has been proposed as a dynamic process which is integral to the formation of an oriented fibre system. A similar mechanism is responsible

for the orientation of connective tissue in vivo (Harris et al. 1981, Stopak et al. 1982), such as is seen in the periodontal ligament (Stopak et al. 1982, Aukhil et al. 1986). The attachment of fibroblasts and fibroblast-like cells to the pre-

viously denuded root surface, followed by orientation of these cells and their connective tissue matrix, may contribute to the new attachment apparatus in cases where periodontal disease has been treated surgically. The examin-

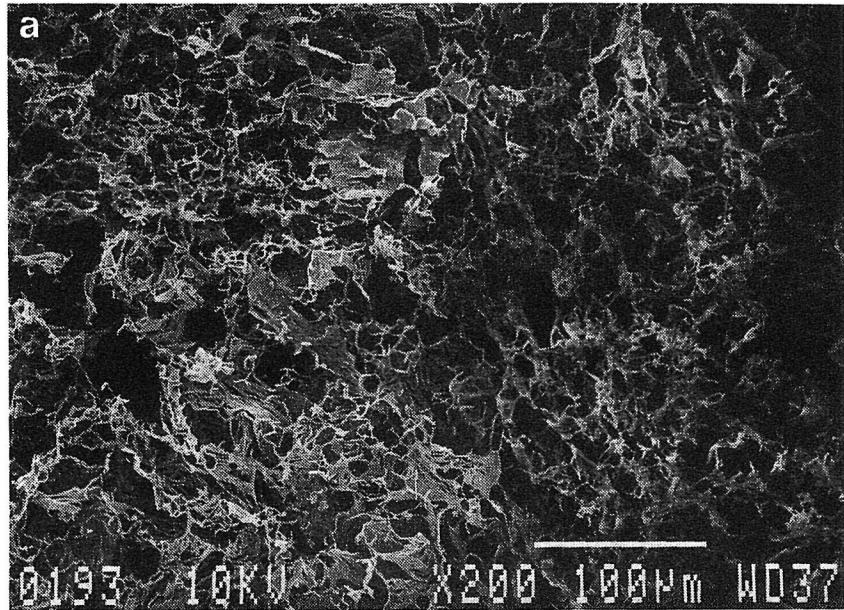


Fig. 4. (a) Low temperature SEM of central portion of vertical section of collagen sponge showing absence of cellular penetration after 1 week in culture (original magnification $\times 200$). (b, c) Cell counts at 7, 14, 28, 42 and 56 days post implantation of the collagen graft material in rat thigh. The points represent the mean and standard deviation of 4 median counts from different specimens. (b) represents the counts from the edge of the implant and (c) the counts from the centre.

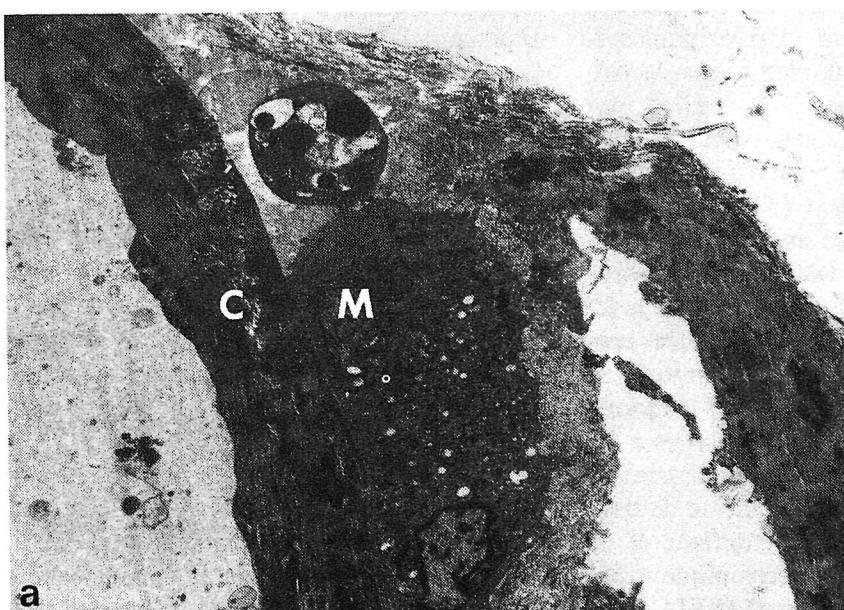
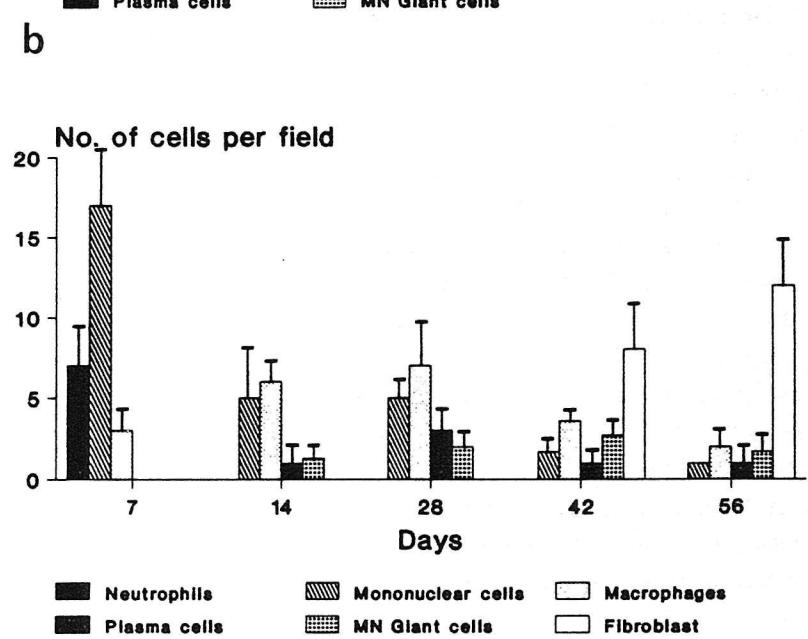
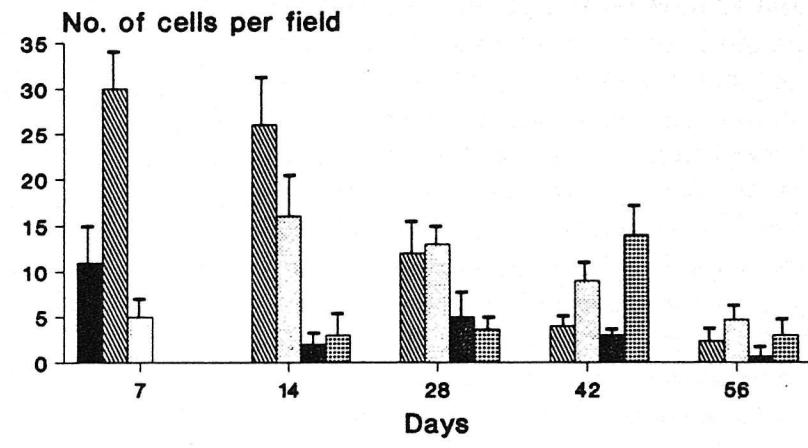
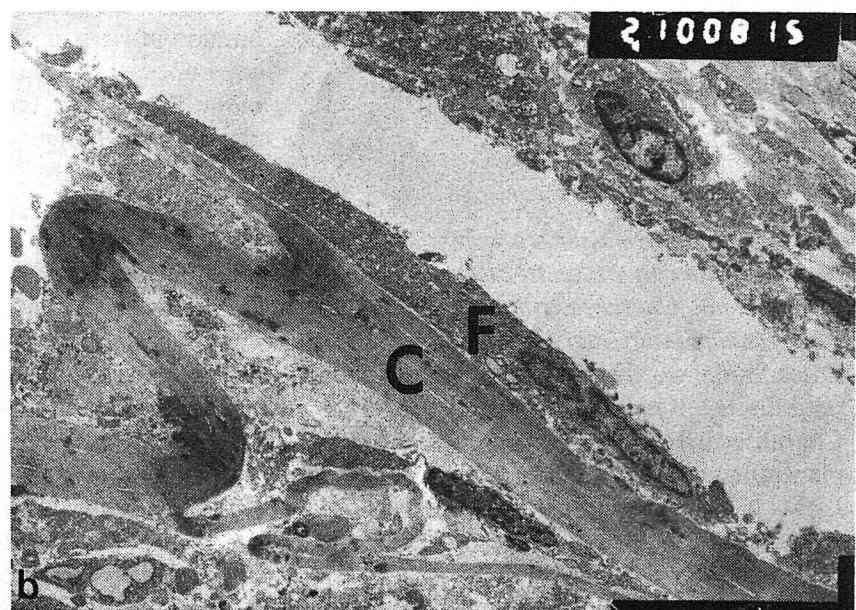


Fig. 5. (a) TEM of collagen sponge implanted in rat for 2 weeks. M = macrophage and C = collagen (original magnification $\times 2000$). (b) TEM of collagen sponge implanted in rat for 6 weeks. F = fibroblasts, C = collagen (original magnification $\times 1800$).



ation of the collagen graft in the rat by light microscopy (LM) revealed, at the 1st week, a marked influx of inflammatory cells dominated by mononuclear cells. This initial inflammatory response may be attributed to the trauma arising from implantation and the chemotactic effect of the collagen material and its peptides. Although the chronic inflammatory reaction persisted between 14 and 42 days post-implantation, with the presence of macrophages, plasma cell and multi-nucleated giant cells, it was minimal at 56 days. The results of in vivo experiments showed a retardation of cellular ingrowth into the graft material over 6 weeks when LM and TEM were used. The retarded cellular ingrowth into the graft material is desirable in the practice of guided tissue regeneration, where it would be detrimental to the performance and durability of the graft. The in vivo specimens showed resorption with fibroblast invasion at 6 weeks, a period which may be long enough for establishment of the new attachment in the periodontal defects of humans.

The methodology of graft preparation for electron microscopy is important in obtaining suitable results. For example those SEM methods which employ fixation and dehydration, inevitably lead to the extraction and dissolution of cell and matrix components causing cell collapse (Thyberg et al. 1973), whereas low temperature SEM employs a rapid freezing technique which minimises loss of water and structural changes of these cells.

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Zusammenfassung

Licht- und Elektronenmikroskopische Bewertung der Biokompatibilitäts-, Resorptions- und Penetrations Charakteristika von humanem Kollagenimplantatmaterial.

Diese Studie wurde initiiert, um die Biokompatibilitäts-, Resorptions- und Penetrations-Charakteristika von humanem Kollagenimplantatmaterial in vitro und in vivo mittels Licht- (LM) und Elektronenmikroskopie (EM) zu testen. Um diese Beziehung zu studieren, wurden Stücke von Kollagenschwämmen ($1 \times 1 \times 0.5$ cm), die durch Glutaraldehyd vernetzt wurden, (1) in sterilen Petrischalen zusammen mit humanen gingivalen

Fibroblasten (HGF) und humanen parodontalen Fibroblasten (HPLF) für zwei Wochen kultiviert und (2) in subkutane Taschen, die in beiden Oberschenkeln (zusammen 20 Taschen) von 10 Sprague-Dawley Ratten gebildet wurden, für 7–56 Tage implantiert. Das Verhalten der Fibroblasten während des Kulturrwachstums wurde im Inversmikroskop (LM) studiert. Anschließend wurden die Gewebekulturproben mit und ohne den Gebrauch eines Tieftemperatur-Rasterelektronenmikroskops (LTSEM) betrachtet. Blöcke, die man von der Implantationsstelle der Ratten gewonnen hatte, wurden für LM und Transmissions-EM präpariert. Die Langzeitbeobachtungen während der ersten 48 Stunden zeigten im LM eine zufällige Orientierung der Zellen der Kultur auf und um die Kollagenschwämme herum. Zwischen 7 und 14 Tagen war die Mehrheit der Zellen, die neben dem Schwamm lagen, im rechten Winkel zu seinem Rand orientiert und lagen mit ihren Längsachsen nahezu parallel zueinander. Das LTSEM zeigte, daß eine große Anzahl an HGF und HPLF auf den Kollagenschwämmen wuchs, aber eine zelluläre Penetration in die Mitte des Schwamms wurde nicht gesehen. LM und TEM der Rattenproben zeigten eine zelluläre Reaktion auf das Kollagenimplantat, als auch eine langsame Resorption und eine Fibroblasteninvasion des Implantats nach 6–8 Wochen. Es wurde der Schluß gezogen, daß humanes Kollagenimplantatmaterial mit HGF und HPLF biokompatibel ist und 42 Tage nach der Implantation die erste Penetration beobachtet wird. In der in vivo Studie erfuhr das Kollagen über einen Zeitraum von 8 Wochen eine langsame Resorption.

Résumé

Caractéristiques de la biocompatibilité, de la résorption et de la pénétration d'un matériau pour greffe à base de collagène humain: évaluation au microscope optique et au microscope électronique

Ce travail a été entrepris pour tester la biocompatibilité, la résorption et la pénétration d'un matériau pour greffe à base de collagène humain in vitro et in vivo à l'aide du microscope optique (LM) et du microscope électronique (EM). Pour étudier cette relation, des morceaux d'éponges collagènes à liaison croisée au glutaraldehyde ($1 \times 1 \times 0.5$ cm) ont été (1) mis en culture pendant 12 semaines dans des boîtes de Pétri stériles avec des fibroblastes gingivaux humains (HGF) et des fibroblastes desmodontaux humains (HPLF), (2) implantés dans des poches sous-cutanées pratiquées dans les 2 cuisses (20 sites en tout) de 10 rats Sprague-Dawley pendant 7–56 jours. Le mode de croissance des fibroblastes a été étudié au microscope optique à lumière inversée (LM), les échantillons de culture de tissu ont ensuite été étudiés de l'extérieur et de l'intérieur au microscope électronique à balayage à basses températures (LTSEM). Des blocs prélevés chez les rats dans les sites des greffes ont été préparés pour LM et pour EM

à transmission (TEM). Les observations LM à long terme mettaient en évidence un attachement et une orientation aléatoire des cellules sur l'éponge collagène en culture et autour d'elle pendant les 48 premières heures. Entre 7 et 14 jours, la majorité des cellules adjacentes à l'éponge étaient orientées perpendiculairement à son bord, avec leurs axes longitudinaux à peu près parallèles les uns aux autres. La LTSEM révélait que de nombreux HGF et HPLF croissaient sur les éponges de collagène, mais on ne constatait pas de pénétration collagène au milieu de l'éponge. Avec LM et TEM, on constatait dans les échantillons provenant des rats une réaction cellulaire envers le greffon collagène, ainsi qu'une résorption lente, et une invasion fibroblastique du greffon aux semaines 6–8. En conclusion, le greffon de collagène humain était biocompatible avec HGF et HPLF; la pénétration n'était pas observée avant le jour 42 après l'implantation. Dans l'étude in vivo, le collagène était le siège d'une lente résorption sur une période de 8 semaines.

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