JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 3 (1992) 239 - 244

Subcutaneous and intramuscular implantation of sepiolite-collagen complexes

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The tissue response after subcutaneous or intramuscular implantation of sepiolite-collagen complexes has been studied. These implants form a continuous three-dimensional matrix, showing a fibrous and rough surface topography. The host tissue response against the collagen-sepiolite complexes is a foreign-body reaction, focally intense, with abundant giant cells, typical of resorbable biomaterials. Sepiolite-collagen complex (SC) appears to be well tolerated and almost entirely resorbed within most of the experimental lesions. Sepiolite-collagen complex cross-linked with glutaraldehyde (SCG) demonstrates a significantly greater resistance to biodegradation than the non-cross-linked product. The anti-bovine collagen antibody levels in the sera of implanted rats were studied. The subcutaneous implantation of SC complexes induces a low immunological reaction, while it is almost negligible for the SCG ones.

1. Introduction

Collagen is the major component of the extracellular matrix and its metabolism is directly associated with many physiological processes involved in biological adaptations and tissue regeneration [1]. Collagen-based biomaterials have been used for a variety of human clinical applications [2–6]. Sometimes a mineral component is involved in the preparation of these products [7]. We have described the interaction of type I collagen and sepiolite (SC complex) [8–10], as well as the treatment of this complex with glutaraldehyde (SCG complex) [11] and their potential uses as biomaterials. Neither of the collagen-sepiolite complexes exhibits any toxicity for human skin fibroblasts in culture [11]. The sepiolite-collagen complexes also allow normal cell attachment, as confirmed through fibroblast adhesion and spreading experiments [12]. The growth, morphology and collagen biosynthesis of human fibroblasts obtained and cultured on sepiolite-collagen complexes are not modified when compared with human fibroblasts cultured under standard conditions [13, 14]. According to these studies the sepiolite-collagen complexes may be useful in the biomaterial field.

However, when extrapolating data from *in vitro* studies to *in vivo* situations a certain amount of caution must always be exercised. For this reason, implantation tests are frequently used to evaluate the biocompatibility of biomaterials [15, 16]. Thus, this work attempts to study the *in vivo* biocompatibility of sepiolite-collagen complexes. The study involves subcutaneous and intramuscular implantation of sepiolite-collagen complexes by using an *in vivo* rat model to assess tissue reaction and fibrous ingrowth.

2. Materials and methods

2.1. Preparation of sepiolite-collagen complexes

Type I collagen from fetal calf skin was purified and characterized as previously described [8, 17]. Sepiolite was kindly supplied by Tolsa S.A. (Madrid, Spain). The sepiolite-collagen complex (SC) was routinely prepared at a 0.6 protein/sepiolite mass ratio, as previously described [8]. Treatment of the complex with glutaraldehyde was performed in 0.1 M phosphate buffer, pH 7.4, containing 0.5% glutaraldehyde for 20 min at room temperature [11]. The treated complexes were exhaustively washed with phosphate-buffered saline to remove potential free aldehyde. Evaluation of protein cross-linking was performed as previously described [18]. The sepiolite-collagen complexes were sterilized and dried under ultraviolet light immediately before use.

2.2. Scanning electron microscopy

Sepiolite-collagen complexes and collagen-coated 12 mm diameter glass coverslips (placed in dishes for handling) were prepared as previously described [12]. All specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 45 min at room

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temperature. Dehydration was performed with slow water replacement by a series of acetone solutions and final dehydration in absolute acetone. Specimens were allowed to dry under vacuum at room temperature. The coverslips were mounted on stubs and coated in vacuum with gold–palladium by using a sputter coater, and observed by scanning electron microscopy (Philips SEM 500).

2.3. Subcutaneous implantation

Wistar strain male rats (150 g each in mass) were used in this study. The number of animals ranged from 10 to 30 for each time point and group considered. Two sterilized sepiolite-collagen implants ($3 \times 2 \times 1$ mm^3) were usually inserted dorsally by aseptic techniques (one each in the scapular and pelvic regions) in rats anesthetized with ether. The implants were inserted subdermally far away from the cutaneous incision to minimize effects of trauma on tissues surrounding the implants. The incisions were sutured by interrupted threads. Only one case of infection was found among 60 studied implantations and it was discarded.

At selected time points, the animals were sacrificed by ether inhalation, and the implants were surgically excised intact.

2.4. Intramuscular implantation

Intramuscular implantations of the sepiolite-collagen implants were made into the gluteus muscle of Wistar strain male rats. The number of animals ranged from 10 to 30 for each time point and group considered. Under ether anesthesia and sterile conditions, a pocket was made in the gluteal muscle separating the fibres longitudinally. After the implant insertion, the fascia, muscle and skin layer were sutured.

At selected time points, the rats were sacrificed and a block of muscle containing each implant was removed.

2.5. Histological methods

For histological examination, the implant sites with the sample were excised and removed with sufficient surrounding tissue. Implants were fixed in 10% neutral buffered formalin for a minimum of 24 h, and embedded in paraffin. Sections (8 μm) were cut and stained with hematoxylin/eosin, Masson's trichrome, and von Kossa stain.

2.6. Humoral immunity

Blood was obtained by cardiac puncture. The serum samples were separated by centrifugation and stored at $-20\,^{\circ}$C until required. Serum antibodies were measured by an Enzyme Linked Immunoabsorbent Assay (ELISA).

The type I collagen from fetal calf skin used in the preparation of the complexes was coated on the bottom of 96 wells polystyrene plates (Costar Europe Ltd, Badhoeredorp, The Netherlands). Peroxidase-conjugated rabbit anti-rat IgG antibodies from Bio-Yeda (Rehovot, Israel) were used in these studies. Control wells were prepared without collagen or without rat serum. The absorbance values were recorded in an automated ELISA plate reader at 492 nm (Titertek Uniskan II, Flow Lab., UK).

3. Results and discussion

The events occurring at the interface of an implanted material with its physiological environment must be considered when the biocompatibility of the implant is studied. Thus, geometry and surface have a direct influence on the biological performance of an implant and can affect both the tissue reaction and enzymatic activity associated with the biomaterial.

The morphology of both SC and SCG complexes and a film of type I collagen has been studied (Fig. 1). Scanning electron microscopy shows that air-dried collagen gels are collapsed and have a smooth surface. In comparison, the sepiolite-collagen complexes form a continuous three-dimensional matrix, showing a fibrous and rough surface topography. The morphology of these structures can mimic the collagenous meshwork present in the extracellular matrix, thus acting as an *in vivo* substitute.

Histological analysis of SC and SCG implants also reveals significant differences in the matrix architecture (Fig. 2). The SCG implants exhibit a more continuous surface than the SC complex, which presents a more amorphous appearance. Similar differences have been observed when the *in vivo* influence of heparin on the matrix organization of fibrillar collagen implants was studied [19]. These differences could result in different tissue response to the implantation of both types of complexes.

3.1. Implantation studies

In vivo tests are required steps in the study of the behaviour of biomaterials. They may be accomplished by monitoring the response after subcutaneous or

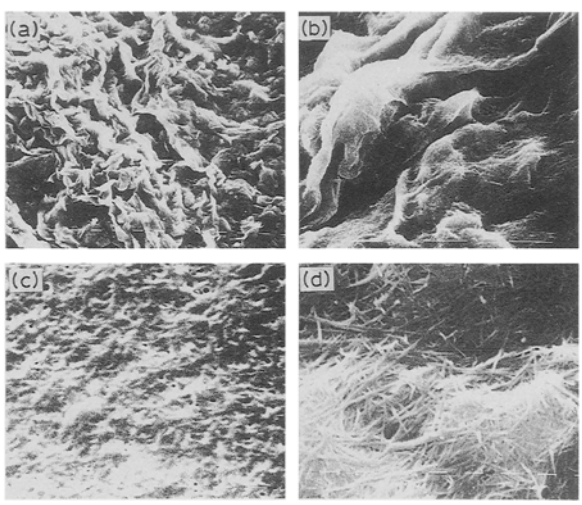

Figure 1 Scanning electron micrographs showing the SC- and SCG-complex surface topography before implantation. SC complex (a, × 175, d, × 2720), SCG complex (b, × 685), and a type I collagen film (c, × 2720).

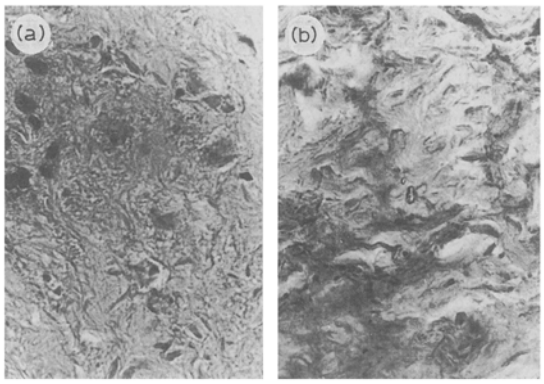

Figure 2 Morphology of sepiolite-collagen complexes after implantation. SC complex (a) and SCG complex (b) were implanted subcutaneously in rats, harvested at seven days post-implantation and stained with hematoxylin and eosin (× 400).

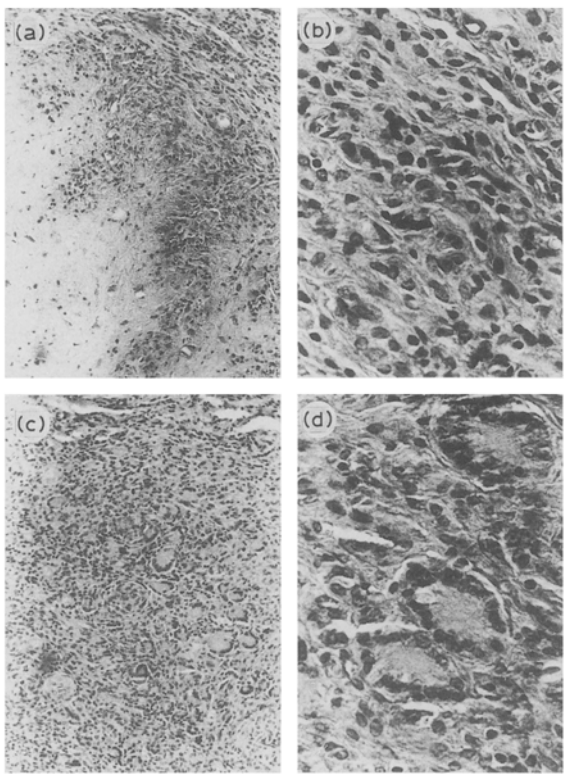

Figure 3 Subcutaneous implantation of SC complex. SC complex was implanted subcutaneously in rats as described. The implants were surgically excised (a, × 100; b, × 400) one week and (c, × 100; d, × 400) four weeks after implantation. (b) and (d) correspond to magnifications of the infiltrated areas. Sections were stained with hematoxylin and eosin.

intramuscular implantation of the considered material for different periods of time. Analysis of inflammatory and healing responses appear as a valid test for toxicity or innocuity of implanted materials based on both past and present experiments [3, 6, 15, 20]. The main part of these studies are based on histologic observations and morphologic evaluations [6, 7]. In general, the observed implant-tissue response is a foreign-body reaction, which varies from minimal to extreme, depending on the nature of the biomaterial. This response must necessarily be considered since the success or failure of a reconstructive procedure mainly depends on the host reaction to the implanted biomaterial.

Figs 3 and 4 show the biological response to SC and SCG complexes after subcutaneous implantation in rats. In brief, the cellular events that occur during the implantation time could be summarized as follows.

The outline of the implant is clearly observed one week after subcutaneous implantation of SC complex (Fig. 3a, b). Acute inflammatory cells (polymorphonuclear leucocytes) are present in the tissue. The implant location is surrounded by loose granulation tissue. Many areas show fibroblasts and connective tissue lying tightly against the implanted material. At four weeks the inflammatory response disappears and the granulation tissue matures. The complex is divided by ingrowing connective tissue. Multinucleated giant-cells also appear at the implant site (Fig. 3c, d). At eight weeks, the number of giant cells and the size of SC complex decrease. This material appears surrounded by dense connective tissue. At 12 weeks, no SC complex is visualized by histological examination of tissue sections at the implanted area, and inflamation is absent.

When the SCG complex is considered one week after subcutaneous implantation, the site is surrounded by a round-cell inflammatory reaction caused by either the incision or the presence of the

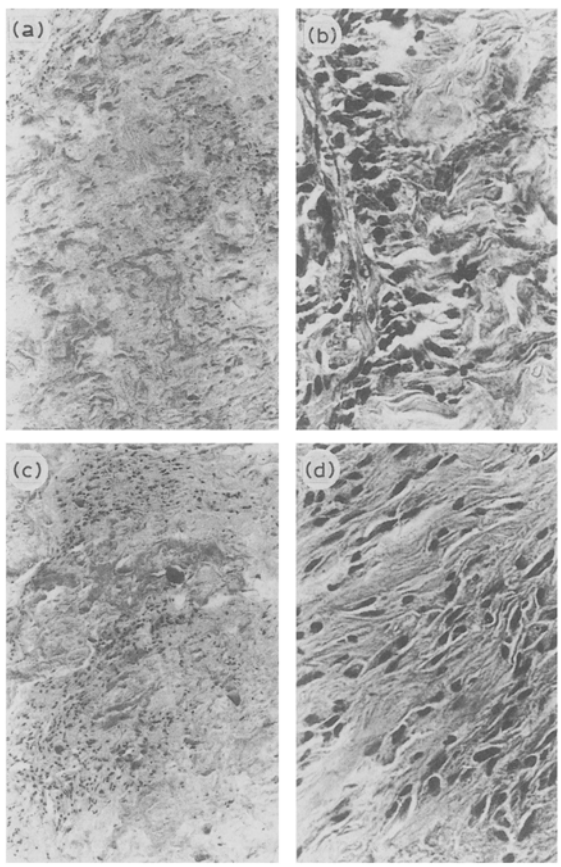

Figure 4 Subcutaneous implantation of SCG complex. SCG complex was implanted as described for SC complex. Histologic sections were obtained after surgical excision of the implants (a, × 100; b, × 400) one week and (c, × 100; d, × 400) three months after implantation. Sections were stained with hematoxylin and eosin.

SCG implant (Fig. 4a, b). At four weeks, the implant zones contain a maturing type of granulation tissue with few, scattered chronic inflammatory cells. A greater presence of collagen is observed. The number of giant cells is lower than for the SC complex. At three months (Fig. 4c, d) no significant resorption of the material is observed. The inflammatory response disappears. The implanted material is surrounded by a fibroblast membrane and connective tissue components. The central region of the implants remains unaltered, without infiltration (Fig. 4c). At four months the response is similar to the one observed at three months of implantation.

These results indicate that sepiolite-collagen complexes are well tolerated by the tissue. Acute inflammatory cells are consistently present in tissue specimens removed seven days after insertion of all the sepiolite-collagen complexes. This response is commonly observed and is probably due to both the natural minimal traumatic response and the presence of the implant [21].

The initial acute inflammatory response evolves into stages of subacute inflammation, diverse granulation tissues and fibrous tissue repair. Various stages of implant fragmentation and phagocytosis by individual macrophages and giant cells are observed (Fig. 3). Host-tissue necrosis does not appear. This inflammatory response is characteristic of a typical resorbable material [22, 23].

The general pattern of cell invasion into non-cross-linked implants (SC) is more extensive than that observed in the cross-linked ones (SCG). This enhanced cell invasion results in a more rapid disappearance of the implanted material. Three months after implantation, the SC implant is completely resorbed while SCG-implant remains mainly acellular and surrounded by a membrane of fibroblasts and connective tissue components (Fig. 4c, d). The reduced resorption of the sepiolite-collagen complex due to glutaraldehyde treatment correlates well with its previously observed enhanced resistance to collagenase degradation [11]. Similar behaviour has been described for other cross-linked materials when compared with their non-cross-linked counterparts [5, 6, 24, 25].

The results indicate that SC complex is well tolerated by the subcutaneous tissue, being a potential resorbable and biodegradable biomaterial. Glutaraldehyde treatment of SC complex increases its resistance to *in vivo* degradation.

The resorption of the collagenous material occurs by the infiltrating inflammatory cells, principally macrophages, and to a lesser extent, granulocytes. Both cell lines synthesize extracellular and intracellular collagen degrading enzymes [25–27].

Cellular response and tissue repair mechanisms after intramuscular implantation of the two biomaterials is similar to that observed after subcutaneous implantation. Fig. 5 shows the extent of the inflammatory response to intramuscular implantation of SC and SCG complexes. The enhanced persistence after intramuscular implantation has been also described for other implants and it can be attributed to the movement of the implant-tissue interphase due to regular muscle contractions [15, 28, 29].

Calcification studies on both SC and SCG implants, performed by von Kossa staining, do not indicate implant calcification (only two cases over 60 different implants).

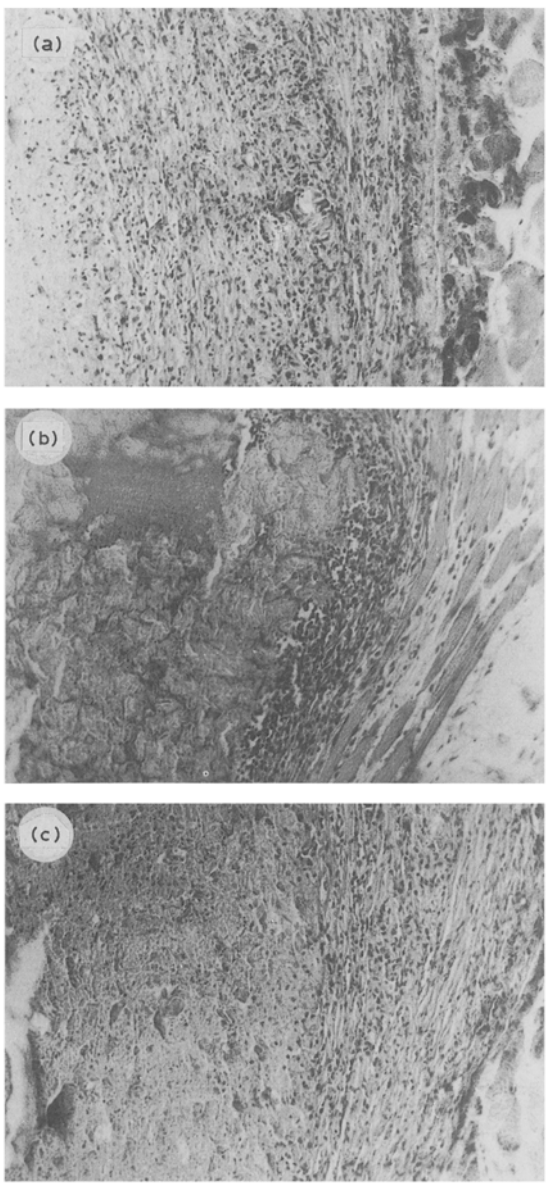

Figure 5 Comparison of the tissue reaction to intramuscular implantation of non-cross-linked (SC) and cross-linked (SCG) sepiolite collagen complexes. Sepiolite-collagen complex intramuscular implantation was performed as described in §2. SC complex (a) four months after implantation; SCG complex (b) one month and (c) four months after implantation. Sections were stained with hematoxylin and eosin (× 100).

3.2. Immunological response

Immune tissue rejection is an important obstacle in using natural products and reconstituted tissue macromolecules as bioprotheses. However, there is no evidence that ceramics, or even sepiolite, induce sensitization [30]. It is also well known that bovine collagen preparations exhibit low immunogenicity [25]; data on the immunogenic potential of collagen biomaterials have been reported in the literature [16, 31–34].

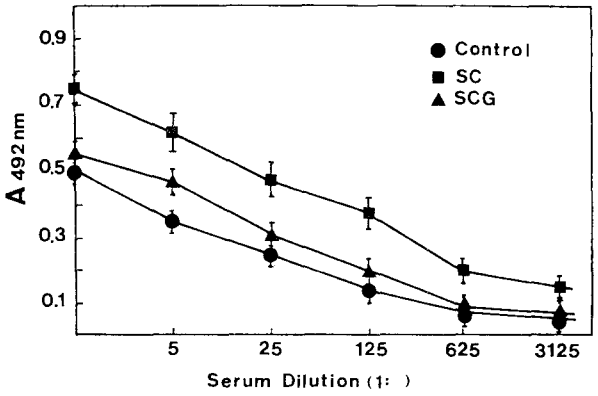

Figure 6 Rat serum anti-collagen antibody titration at two months after SC- and SCG-complex implantation. Sera from SC-complex (■) and SCG-complex (▲) implanted rats were compared to those from controls (●), for antibodies to type I collagen. ELISA data are presented as the mean absorbance values ± SD (averages of eight points for each dilution and animal) for the 30 animals considered in each studied group.

In any case, the immune response to implantation of different types of sepiolite-collagen complexes was studied. The absence of plasma cells, after morphological examination, suggests that there is no significant humoral immunological activity [21].

Sera obtained after implantation of sepiolite-collagen complexes were examined for antibodies against bovine type I collagen by using the ELISA tests (Fig. 6). The immunological response after subtraction of the control values is low. According to these results, subcutaneous implantation of sepiolite-collagen complexes induces a low immunological reaction, which confirms that the characteristic structure of collagen makes it poorly immunogenic. On this idea, Oliver *et al.* [35] have demonstrated that transplanted dermal collagen is resorbed by non-specific digestion rather than by direct cell or humoral-mediated immune processes.

Different studies have demonstrated that glutaraldehyde cross-linked bioimplants enhance *in vivo* survivability and diminish immunogenicity [16, 25]. Our data demonstrate that the antibody titres to fetal bovine type I collagen were significantly decreased in the sera of SCG-implanted rats when compared to SC-implanted animals, and were similar to that obtained for control rats, although being always very low. These data indicate that the treatment with glutaraldehyde of the SC complex decreases its immunogenicity down to minimal values.

These studies constitute the next step in assessing the potential use of sepiolite-collagen complexes in the design of bioprotheses. The observed *in vivo* resorbable character of these complexes as well as their tolerance by the surrounding tissue are positive results towards this overall goal.

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Received 11 December 1990
and accepted 25 April 1991