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EFFECT OF ALDEHYDE CROSS-LINKING ON HUMAN DERMAL COLLAGEN IMPLANTS IN THE RAT

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Summary.—The fate of s.c. implants of fibrous, trypsin-purified human dermal collagen and collagen cross-linked with formaldehyde and glutaraldehyde has been studied in rats. Dermal collagen, and untreated skin implants, underwent resorption associated with a pronounced round-cell reaction. While collagen implants cross-linked with solutions of 0·04% and 0·08% formaldehyde became reduced in size, those cross-linked with 1% formaldehyde and 0·01%, 0·02% and 0·04% glutaraldehyde, although undergoing some collagen remodelling, retained their original size over the 25-week period of study. At 5 weeks the aldehyde cross-linked implants showed their greatest cellularity, reaching a lower, more stable cell population by 18 weeks. More round cells were seen at 5 weeks, particularly after formaldehyde cross-linking, than at later times when few were present. The results indicate that aldehyde-stabilized preparations of heterograft dermal collagen could have applications in the repair of tissue defects in man.

THERE IS INCREASING INTEREST in the use of collagen-based preparations for the replacement of a variety of soft body tissues (*e.g.* Knapp, Kaplan and Daniels, 1977; McGregor and Lindop, 1974; Rose, 1972).

The feasibility of using trypsin-purified, allogeneic skin collagen for the repair of skin defects has also been demonstrated, both as s.c. implants in animals (Oliver, Grant and Kent, 1972; Oliver *et al.*, 1976) and man (Shakespeare and Griffiths, 1980) and in the reconstruction of full-thickness-loss skin wounds in rats (Oliver *et al.*, 1977; Oliver and Grant, 1979). If heterograft skin collagen could be similarly employed this might prove to be of great clinical value. To assess this possibility a study has been made of trypsin-purified human skin collagen when implanted s.c. in rats. This included collagen implants cross-linked with a range of low concentrations of glutaraldehyde or formaldehyde, a procedure known to introduce extra stability in collagen grafts (Oliver *et al.*, 1976) and heart valve prostheses (Woodroof, 1978).

To help determine the host response, particular attention was given to the cellularity of the implants over the 25-week period of study.

MATERIALS AND METHODS

Twenty-one inbred hooded rats (PVG/C strain) of both sexes and aged 5–6 months were used as recipients.

Thin strips of full-thickness human abdominal skin were kindly made available by arrangement with Mr J. C. Forrester, Ninewells Hospital, Dundee, for implantation either as untreated skin or dermal collagen grafts.

Preparation of dermal collagen.—Cell-free, dermal collagen was prepared for implantation by incubating the skin in a solution of crystalline trypsin (Koch–Light; 2 mg/ml in 0·1M phosphate buffer solution, pH 9·0, with 0·5 mg/ml sodium azide as a bactericide) at 15° for 28 days as previously described (Oliver *et al.*, 1977).

To determine the effects of this enzymic treatment on human skin, specimens treated 1–28 days in the trypsin solution were fixed for light and electron microscope examination as previously described (Oliver *et al.*, 1976).

Aldehyde treatment.—Solutions of glutaraldehyde (Taab Laboratories) or formaldehyde (Analar: British Drug Houses) were diluted with 0·1M phosphate buffered saline, pH 7·2, to appropriate concentrations immediately before

use. Preparations of dermal collagen (100 mg/ml) were incubated in the aldehyde solutions for 16 h at 15° before washing for 4 h in several changes of normal saline and placed in "199" culture medium (Difco) prior to grafting.

Operation.—Anaesthesia was induced with ether and one flank closely shaved, then swabbed with Hibitane. Skin and dermal collagen preparations were implanted s.c. in dissected pockets beneath the panniculus carnosus.

The following implants, all of which measured 1·5 × 0·5 cm and approximately 0·3 cm in thickness, were made: 4 untreated skin (biopsied at 3–16 weeks); 8 dermal collagen (biopsied at 3–24 weeks) and 6 groups of 5 dermal collagen. Each group was treated in one of the following aldehyde solutions: 0·01, 0·02 and 0·04% glutaraldehyde and 0·04, 0·08 and 1% formaldehyde. The aldehyde-treated implants were biopsied at 5, 10, 14, 18 and 25 weeks.

At biopsy the skin was raised, the adherent implant, where present, measured and the complete implant and overlying skin fixed in 10% formol saline. Each implant was cut longitudinally, wax-embedded, serially sectioned at 8 μm and stained with haematoxylin and eosin.

Cell counts.—An indication of the cellularity of the aldehyde-treated implants was obtained in the following way: for each implant counts were acquired from 2 sections, 1 or 2 sections apart, using a field diameter of 0·23 mm; counts were made at 4 equally spaced points on the periphery and at 2 equally spaced points in the middle of the implants; thus for any given implant peripheral cell numbers represent the mean ± s.d. of 8 counts and central numbers the mean ± s.d. of 4 counts.

RESULTS

Trypsin treatment of human skin

The cellular components of the skin had virtually disappeared by 4 days and continued treatment left a cell-free, fibrous preparation composed of collagen (and elastin) which retained its original bundle architecture. The electron-microscope study confirmed that progressive removal of structureless nonspecific debris occurred without alteration to the collagen fibrils.

Skin and dermal collagen implants

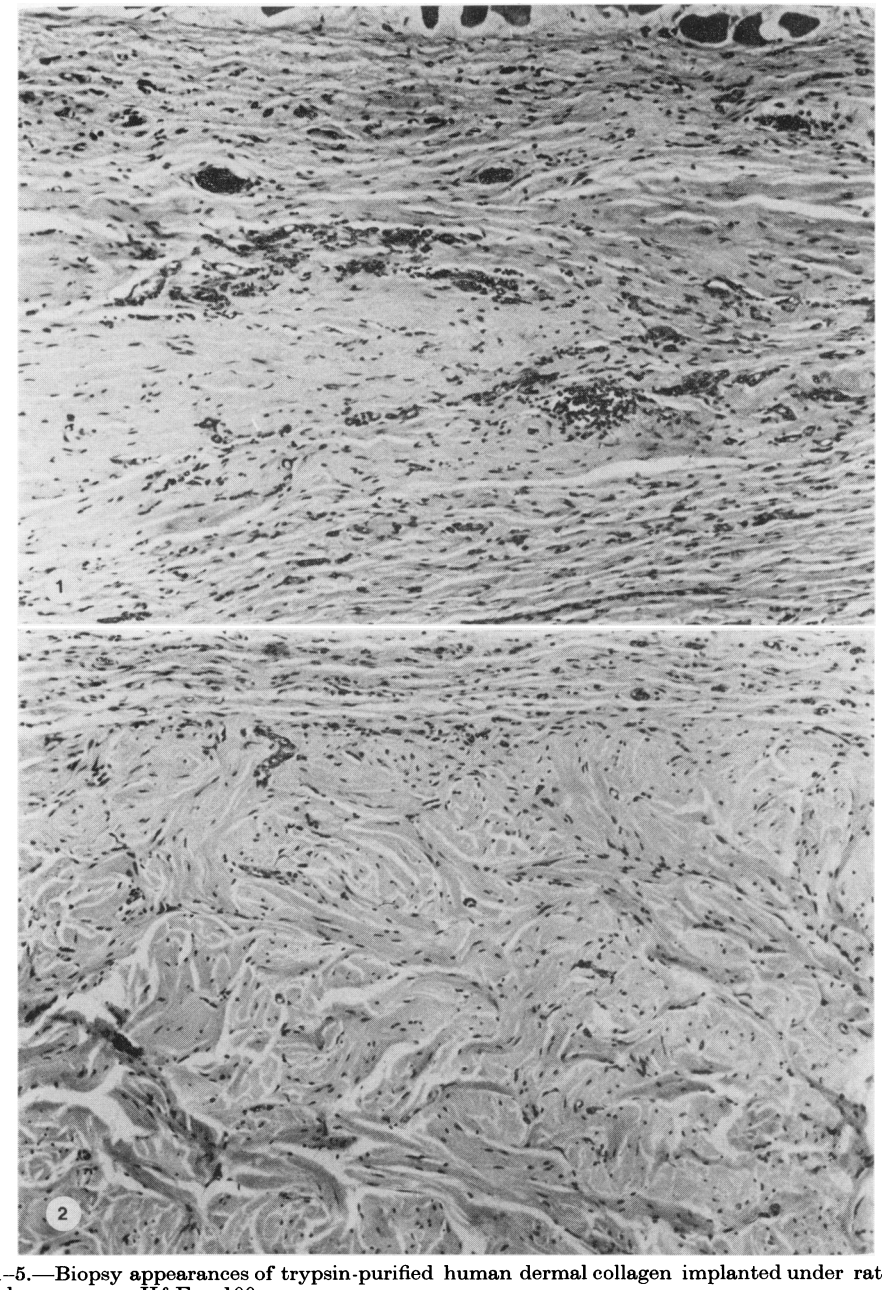
Implants of untreated human skin were resorbed by 16 weeks. The trypsin-purified dermal collagen implants appeared intact up to 6 weeks when they were recellularized and revascularized. However, from 9—24 weeks the implants underwent continuing resorption, becoming greatly reduced in thickness, and at 24 weeks measured less than a third of their original areas. Histological examination revealed gross collagenolysis associated with a pronounced mononuclear infiltrate (Fig. 1).

Aldehyde-pretreated implants

All of the glutaraldehyde-treated and the 1% formaldehyde-treated implants retained their original size over the 25-week period of study. However, the 0·04% and 0·08% formaldehyde-treated implants became thinner and showed a reduction in size by approximately 25% at 25 weeks.

At 5 weeks all of the implants were colonized by cells, revascularized and ensheathed in a thin layer of connective tissue (Figs 2 and 3). In general, at this time, the implants showed their greatest cellularity (Tables I and II), reaching a lower, more stable cell population by 18 weeks. Initially a higher density of cells was seen in implants treated with 0·02% and 0·04% glutaraldehyde and 1% formaldehyde than in the 0·01% glutaraldehyde-treated implants. The higher peripheral counts, as compared with the central counts, were due to two factors: throughout there was a higher degree of vascularization and greater numbers of fibroblasts at the periphery; in addition, particularly in the 5-week biopsy specimens, there was a greater peripheral cellular reaction. In all of the implants a more pronounced, but variable, round-cell infiltrate was observed at 5 weeks than at later times, when few mononuclears were present. This was particularly apparent in the formaldehyde-treated implants (*cf.* Figs 2 and 3).

By 14 weeks most of the implants showed evidence of peripheral collagen remodelling and thickening of the collagen bundles. While at 18 weeks the glutaraldehyde-treated implants showed good graft preservation (Fig. 4), with time the original collagen architecture of all implants tended to become altered (Fig. 5) regardless of whether the implants underwent gross loss of size or not. Those that



Figs 1–5.—Biopsy appearances of trypsin-purified human dermal collagen implanted under rat panniculus carnosus. H&E × 100.

FIG. 1.—Collagen implant undergoing resorption and showing a round-cell infiltrate, 15 weeks.

FIG. 2.—Collagen implant, cross-linked with 0·02% glutaraldehyde, 5 weeks. The implant is recellularized and revascularized and ensheathed in a thin layer of connective tissue.

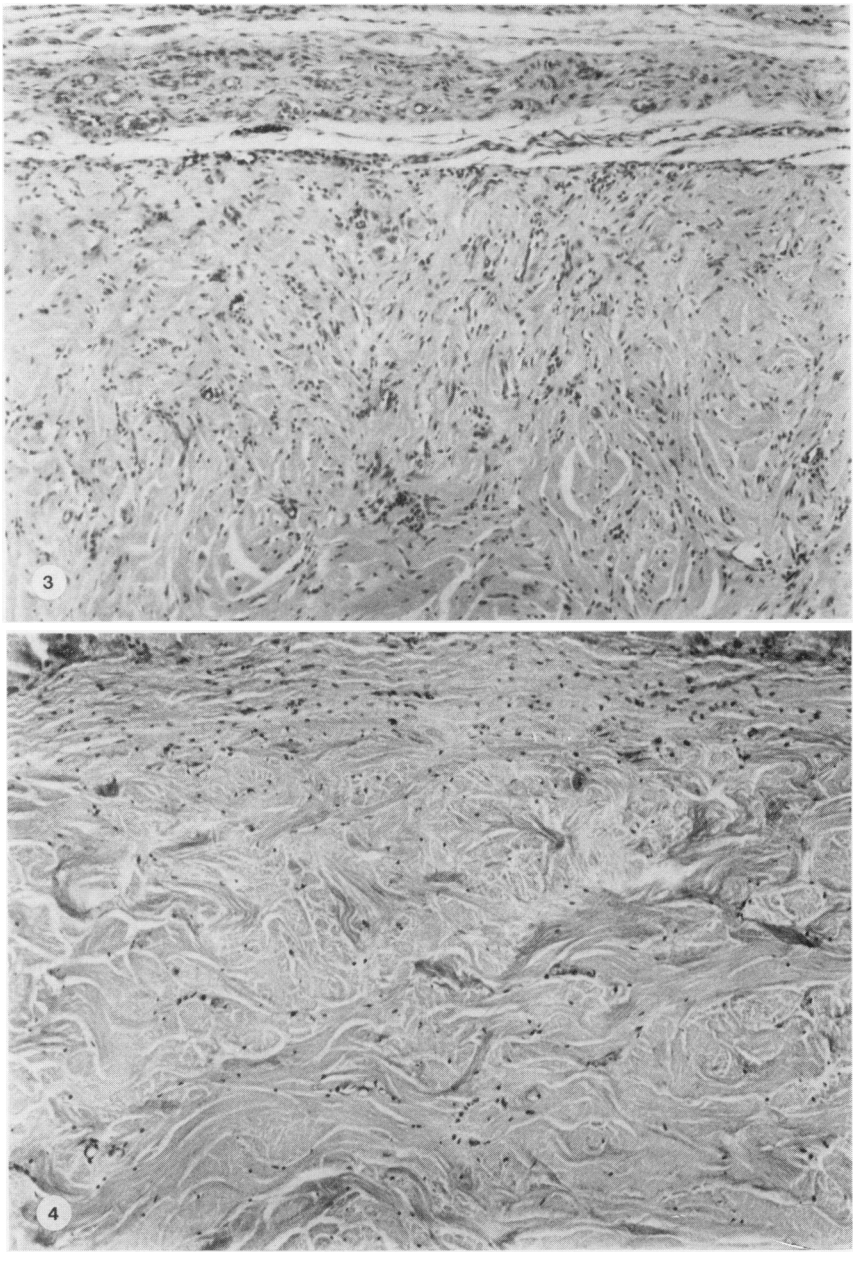


FIG. 3.—Collagen implant, cross-linked with 1% formaldehyde, showing a peripheral inflammatory response at 5 weeks.

FIG. 4.—Collagen implant, cross-linked with 0·02% glutaraldehyde, 18 weeks. The implant is sparsely cellularized and shows preservation of the original collagen bundle architecture and fibrous union with the surrounding connective tissue.

TABLE I.—*Cell counts (± s.d.) at the periphery of aldehyde-cross-linked collagen implants*

Concentration of aldehyde	Time of biopsy in weeks			
	5	10	18	25
0·01% Glutaraldehyde	53·1 ± 10·5	46·3 ± 14·8	30·3 ± 12·8	37·0 ± 18·5
0·02% Glutaraldehyde	172·1 ± 20·7	53·3 ± 24·2	37·3 ± 13·0	41·4 ± 12·7
0·04% Glutaraldehyde	134·5 ± 62·2	62·3 ± 21·6	28·0 ± 12·6	40·6 ± 10·2
1% Formaldehyde	174·5 ± 61·8	61·1 ± 23·4	61·0 ± 29·9	47·4 ± 27·1

Counts were obtained from 4 points at the periphery of each implant as described in MATERIALS AND METHODS.

TABLE II.—*Cell counts (± s.d.) at the centre of aldehyde-cross-linked collagen implants*

Concentration of aldehyde	Time of biopsy in weeks			
	5	10	18	25
0·01% Glutarladehyde	12·3 ± 7·4	38·5 ± 19·8	8·0 ± 5·7	15·0 ± 2·5
0·02% Glutaraldehyde	70·0 ± 6·9	16·0 ± 10·5	9·8 ± 2·6	10·5 ± 1·3
0·04% Glutaraldehyde	53·0 ± 12·4	13·8 ± 7·8	10·8 ± 3·8	13·3 ± 6·1
1% Formaldehyde	84·0 ± 27·7	23·8 ± 7·8	19·8 ± 9·1	10·0 ± 2·1

Counts were obtained from 2 points at the centre of each implant as described in MATERIALS AND METHODS.

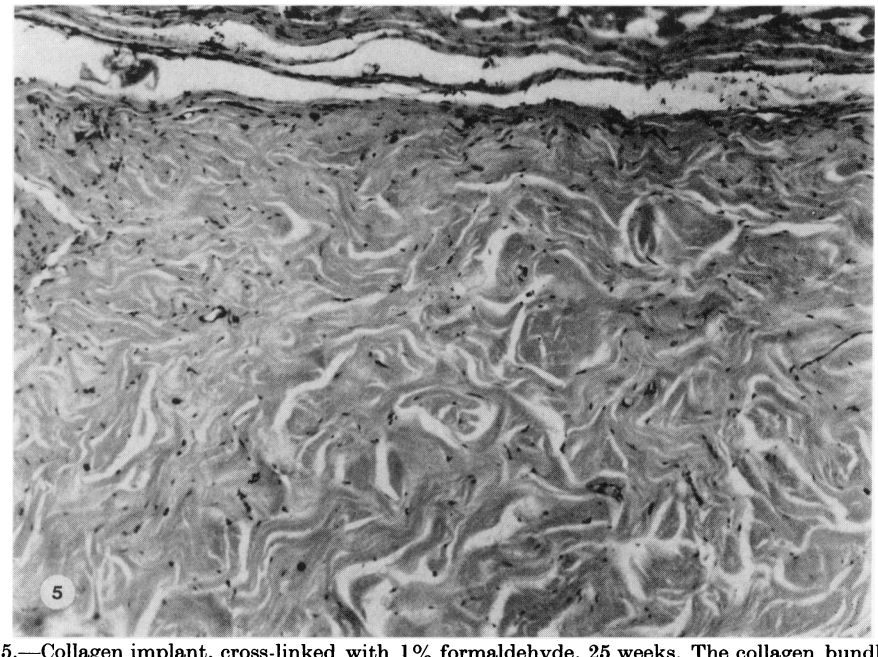


FIG. 5.—Collagen implant, cross-linked with 1% formaldehyde, 25 weeks. The collagen bundles are thickened and show loss of their original organization.

did, following treatment with 0·04% and 0·08% formaldehyde, also showed the presence of macrophages both within and adjacent to the implants. At 25 weeks s.c. nerve fibres were seen to be enclosed within the outer region of several of the implants.

DISCUSSION

Examination by light and electron microscopy of the effect of the trypsin-purification procedure on human skin demonstrated that, while the collagen (and elastin) fibres remained unaltered, all of

the cellular components which have been strongly implicated in skin graft rejection were eventually removed, as was found previously with rat skin (Oliver *et al.*, 1976).

Unlike implants of allogeneic rat collagen, which although undergoing some collagen remodelling largely maintained their original size (Oliver *et al.*, 1976), the human collagen implants eventually underwent resorption associated with a prominent mononuclear reaction. In contrast implants pre-treated with 0·01–0·04% glutaraldehyde and 1% formaldehyde appeared to resist lysis and showed no evidence of a persisting cellular immune response. Increased graft stability and suppression or reduction of tissue antigenicity following aldehyde cross-linking have also been described for heart valves and other tissues (for a review see Woodroof, 1978).

Although numerous studies have shown that formaldehyde and glutaraldehyde react with collagen to produce both intramolecular and intermolecular cross-linkages and hence greater stability, the reaction mechanisms remain ill-understood (see for example Bowes and Cater, 1968; Cox, Grant and Kent, 1973). However, it is known that a greater number of, and more stable, cross-links are formed with glutaraldehyde than with formaldehyde. This was clearly demonstrated in the present study in which a decrease in size occurred in the 0·04% and 0·08% formaldehyde-treated implants. Similarly a loss in mass in 0·1%, but not in 1%, formaldehyde-treated pig collagen implants in rats has also been observed (Barker *et al.*, 1980).

The initially greater cellular reaction seen with the 0·02% and 0·04% glutaraldehyde- and 1% formaldehyde- as compared with the 0·01% glutaraldehyde-treated implants may reflect increased molecular changes in the collagen and/or toxic effects of unbound or loosely bound aldehyde not removed by the washing procedure employed. A recent study using ^{14}C-labelled formaldehyde showed that formaldehyde was lost from pig collagen over a 6-week period before formaldehyde/collagen stability was reached (Barker *et al.*, 1980).

While all of the implants underwent some collagen remodelling most retained their original size and maintained a stable population of cells without an adverse cellular reaction. These results strongly suggest that aldehyde-cross-linked preparations of trypsin-purified heterograft collagen could have considerable potential in the reconstruction of skin and other soft-tissue defects in man.

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