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In vivo degradation of collagen–vicryl materials in rabbit ear chambers

I. N. BIRD*, I. A. SILVER
Department of Pathology, School of Medical Sciences, Bristol BS8 1TD, UK
S. D. GORHAM
Devro Ltd, Moodiesburn, Chryston, Glasgow G69 0JE, UK
D. A. FRENCH
Ethicon Ltd, Bankhead Ave, Sighthill Industrial Estate, Edinburgh EH11 4HE, UK

The *in vivo* breakdown of collagen–vicryl composites (types 9 and 12) was followed in rabbit ear chambers over a period of 5 weeks. In this time the implants were incorporated into the granulation tissue growing in the chambers. Materials that were sterilized by gamma irradiation were resorbed faster than those sterilized with ethylene oxide or disinfected with alcohol, which suggests that irradiation weakened the material. Histological observation revealed that the collagen component had disappeared from all the composites by day 35 and the surrounding tissue showed a mild foreign-body reaction.

1. Introduction

Over the last decade there has been considerable development of collagen-based prostheses for a wide range of surgical applications including haemostasis [1–5], burn and wound dressings [5–10], blood-vessel repair and vascular grafts [11–14], correction of dermal contour deformities [15–17] and hernia repair [18, 19]. Such prostheses have the advantages of being biodegradable, of low antigenicity, non-cytotoxic and in many cases able to support new tissue growth.

One material originally developed for urological surgical applications consists of a collagen film reinforced with a water-soluble vicryl (polyglactin) mesh. The composite is constructed entirely from biodegradable components, is leakproof, possesses a high initial tensile strength, is flexible and easy to suture and has been used successfully to repair partial nephrectomies and full thickness defects of the urinary bladder in rabbits [20–22]. In addition, it has recently been used to reinforce oesophageal anastomoses [23].

Rabbit ear chambers provide a very convenient method for *in vivo* observation of the interaction of host tissue with implanted foreign materials. Evidence of any cytotoxicity or adverse tissue reaction to the implant can be readily obtained in longitudinal studies, as each specimen can be monitored visually over a long period of time, without the sacrifice of large numbers of animals, and reduces the problems associated with animal to animal variability, as each animal can act as its own control.

The purpose of this study was to observe the breakdown of two types of collagen–vicryl composite (types 9 and 12) *in vivo* in rabbit ear chambers, and to determine whether different sterilization/disinfection procedures altered the breakdown in any way.

2. Materials and methods

2.1. Rabbits

Nine first-generation half-lop rabbits were obtained from a commercial supplier. The animals were 5–7 months old and at the time of ear chamber insertion weighed 2.9–3.7 kg. They were kept and used in accordance with UK Home Office regulations under the Animals (Scientific Procedures) Act 1986.

2.2. Ear chamber manufacture

Ear chambers were manufactured in the Department of Pathology, University of Bristol to the design reported earlier [24, 25]. The chambers, consisting of a body (21 mm o.d.) machined down into a "plug", were produced on a lathe from destressed clear polycarbonate plastic dowling. Holes were drilled in the underside of the body to allow tissue to grow into the chamber. A stretched PTFE spacer (thickness 125 μm) was inserted into the recess in the body to delimit the depth of the chamber and the top was sealed with a Melinex (polyester 9) coverslip held in position by a circlip. A nylon "skirt" (32 mm o.d.) was attached to the underside of the chamber with polycarbonate dissolved in methylene chloride. When in position in the rabbit ear the chamber body and plug provided a clear light path through which it was possible to observe the ingrowth of tissue and its subsequent interaction with any implanted materials.

2.3. Sedation

The rabbits were sedated by an intramuscular (i.m.) injection ($0.5\ \mathrm{ml\,kg^{-1}}$) of the neuroleptanalgesic Hypnorm (Janssen Pharmaceutica, Beerse, Belgium).

* *Present address*: Yamanouchi Research Institute (UK), Littlemore Hospital, Oxford OX4 4XN, UK.

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Usually a single dose was sufficient for the purposes of chamber insertion but in exceptional circumstances a further 0.5–1.0 ml was administered. The animals were also sedated when materials were inserted into established chambers and when chambers were removed. No sedation was required when the chambers were being inspected *in situ.*

2.4. Insertion of ear chambers

The method used was a slight modification of that reported previously [25]. Briefly, the rabbit ear was depilated using surgex, a depilatory cream. The cream was applied in a thick layer to the ear hair, left on for 2–3 min and then washed off with plenty of water. Care was taken not to allow any water to run down into the external ear canal. After the ear was thoroughly dried, sites were selected for insertion of chambers such that minimum damage was done to the local vasculature.

In some animals it was possible to insert two chambers per ear, whilst in others there was room for only one. A total of 23 chambers were inserted. A small incision through the skin was made at a suitable site on the inner aspect of the ear. The skin around the incision was freed from the underlying cartilage to a radius of about 1.5 cm, using a flat chisel-edged spatula. This produced a skin pocket of the same diameter as the "skirt" of the chamber. A hole was cut through the ear with a punch (6.25 mm o.d.) at the centre of the skin pouch. The plug of the chamber was inserted through the ear such that the chamber body was located on the inner surface of the ear.

Prior to insertion, the chamber body was filled with sterile phosphate-buffered-saline (PBS) containing penicillin 10 000 units ml^{-1}. By use of a spatula the nylon mesh skirt attached to the underside of the chamber was slid under the skin into the pouch which had been formed previously. Any excess skin was removed from around the chamber body to ensure that it fitted tightly. Thus the chamber sat embedded in the ear, firmly held in place by the skirt and the "ear plug". After insertion of all the chambers the rabbit was given an i.m. injection of long-acting penicillin (Duphar Vetinary Ltd, Southampton, UK).

Careful postoperative monitoring of the chambers was carried out and any exudate was expressed from the skin pouch around the chamber. The plug was gently cleaned with swabs and the area washed with 0.5% Hibitane in 20% alcohol. Any chambers that developed air bubbles were opened by removal of the coverslip, the air expelled with sterile PBS containing penicillin and the chamber resealed with a new sterile coverslip, spacers and circlip.

2.5. Insertion of the materials

The circlip, coverslip and sealing washer were removed. A precut piece of collagen/vicryl composite (3 mm × 5 mm) was inserted into the centre of each chamber which had an established bed of ingrowing granulation tissue. Generally, insertion of material was carried out 10–14 days after the chamber had been placed in the rabbit ear. At this stage a ring of granulation tissue was just visible in the light path through the chamber. Pieces of each material were examined in at least three different chambers and at least two different animals.

2.6. Manufacture of the collagen–vicryl composites

Materials were prepared as described previously [21]. Type 9 and type 12 vicryl (polyglactin) were manufactured from knitted vicryl yarn containing vicryl filaments (2 denier per filament). Type 9 vicryl consisted of a knitted mesh with triangular holes 0.61 mm (base width) × 0.66 mm (base to apex). The type 12 vicryl (see Fig. 1) was knitted into a hexagonal mesh with holes 3.44 mm (long axis) × 2 mm (short axis). The meshes were placed on to a collagen film prepared from defatted bovine hide collagen and then a layer of degassed collagen slurry was gently layered on to the vicryl, which produced a collagen–vicryl–collagen sandwich. The final thicknesses of the type 9 and type 12 composites were 200 and 490 μm, respectively. After drying, the composite was removed and sealed in aluminium foil. The materials were sterilized in two different ways: (1) gamma irradiation, 2.5 Mrad from a ^{60}Co source and (2) ethylene oxide using a Department of Health-approved industrial procedure for surgical products. Residual levels of ethylene oxide present in the materials after sterilization were well below the maximum allowed by the approved procedure. Samples of each material were also disinfected with 100% Analar grade ethanol for 30 min followed by multiple rinses in sterile PBS.

2.7. Ear chamber photography and observation

Chambers were inspected on days 3, 7, 10, 14, 17, 21, 24, 28, 31 and 35 after insertion of the material. In a few cases materials were removed 10 and 17 days after insertion. Each chamber was examined visually for any gross signs of damage or infection and then the rabbit was placed adjacent to a Wild stereomicroscope. The plug on the bottom of the ear chamber was inserted into a hole in a block of perspex mounted on the microscope stage. Tissue ingrowth and destruction of the implants was observed through the chamber light path at magnifications of ×12, ×25 and ×50. Chambers were photographed on each day of observation at the various magnifications, using an Olympus OM 2 camera body attached to one of the eyepieces. Illumination was provided by a ring flash automatically controlled via the camera sensor. Observations were recorded on 35 mm Fuji 64T colour film.

2.8. Removal of chambers and histology

At the end of the observation period (in most cases 35 days) the ear chambers were dissected out of the ear, the coverslips removed and the opened chambers placed in fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3) for at least 24 h. After fixation, the implant remains were dissected out of each

chamber and processed into wax for light microscopy. Sections (6 μm thick) were cut and stained with haematoxylin and eosin (H and E). Slides were inspected and photographed on a Zeiss Ultraphot microscope.

2.9. Electron microscopy

In some cases, dissected tissues from chambers at 35 days were dehydrated and processed into Epon. "Gold" (90–120 nm) sections were cut, mounted on to copper grids, stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope.

3. Results

3.1. *In vivo* observation of implant destruction

The resorption of types 9 and 12 vicryl–collagen composites was studied over a period of 35 days. During this time the tissue fronts in the chambers grew in radially towards the centre, incorporating the implant into the new granulation tissue. The collagen–vicryl composites under test were well tolerated and did not elicit adverse tissue reactions in any of the chambers monitored in this study. No differences in the resorption rate of the type 9 composite compared to the type 12 composite were detected between materials which had been sterilized in the same manner.

A comparison of materials which had been sterilized in the three different ways revealed two distinct patterns of resorption. The first was observed with composites sterilized with ethylene oxide (EO) or disinfected with alcohol. A typical sequence is shown in Fig. 1. By day 3 the implanted materials were shrouded in a layer of inflammatory cells, which disappeared between days 14 and 21, to show a clear outline of the vicryl mesh left embedded in the growing granulation tissue. The mesh continued to remain visible in the tissue to the end of the study (35 days). The second pattern of breakdown was observed with composites which had been gamma-irradiated. Such implants were also covered with a layer of inflammatory cells 3 days post-implantation but these did not disappear until 21–28 days after implantation. The vicryl material that was revealed when the inflammatory cells cleared from these chambers was no longer an integral mesh, as seen with the EO-sterilized and ethanol-treated materials, but fragmented, with 50%–70% of the structure damaged. In all cases the mesh remaining in the chamber was fainter in outline than equivalent implants sterilized by the other methods. Together these observations suggest that gamma irradiation of the composite increased its rate of resorption *in vivo*.

3.2. Histology of ear chamber tissue

Generally the histological picture was very similar for the two types of composite. Analyses of chamber tissues 35 days after implantation revealed that the vicryl component of the implants was incorporated into the granulation tissue (see Fig. 2a). However, at this time, the collagen film associated with the vicryl mesh had disappeared in the chambers studied.

The granulation tissue around the residual vicryl mesh appeared normal and well vascularized. New collagen was observed being deposited by fibroblasts adjacent to the implanted material (Fig. 2b). There were also inflammatory cells still associated with the vicryl material, some of which could be observed boring between vicryl strands within the fibres and forcing them apart. Other cells were grouped in the interfibre spaces within the mesh. Much of the vicryl mesh had cells adhering to it and occasional giant cells were seen associated with the vicryl in both the 17 and 35 days sections, indicative of a mild foreign body reaction. In most of the sections the histological picture was the same. A major difference was noted in the quantity of residual vicryl seen in those composites which had been gamma irradiated. In the ethylene oxide-sterilized and alcohol-treated samples the vicryl remaining at 35 days was still in large bundles of fibres, whereas by contrast, little remained in sections taken from tissue containing composites which had been gamma-irradiated.

As stated above, there was no residual collagen film identifiable in any of the composites 35 days after implantation. Sections taken from implanted material removed at days 10 and 17 (Fig. 3) revealed collagen film around the vicryl mesh heavily infiltrated by inflammatory cells, predominantly macrophages and polymorphonuclear leukocytes. Inflammatory cells either singly or in groups were often associated with holes in the collagen, suggesting that they were directly involved in the destruction of the collagen component of the implant. By day 17 the dissolution of collagen was advanced, with the film split away from the vicryl mesh. The collagen was also being separated into fine strands by the infiltrating cells.

3.3. Electron microscopy

A number of the observations already noted above were confirmed by this technique. The vicryl mesh was fully integrated into the granulation tissue by the end of the study. Cells associated with the implant were typically macrophages, fibroblasts and the occasional foreign body-type giant cell. In some cases fibroblasts were engaged in collagen fibrillogenesis and new collagen was being laid down adjacent to the individual vicryl fibres (Fig. 4).

In all areas of the sections where vicryl was located, cells were in intimate association with it. In cases where the strands lay in the plane of observation, cell pseudopodia were clearly seen extending along vicryl filaments in the fibre bundles (Fig. 5). Such pseudopodia formed intimate junctions with pseudopodia extending from other parts of the strand. There was no evidence of phagocytosis of pieces of the vicryl mesh (in the sections studied), or of any obvious discharge of cell vacuoles on to the surface of the vicryl strands.

4. Discussion

In this study, the *in vivo* degradation of collagen–vicryl composites has been investigated. The destruction

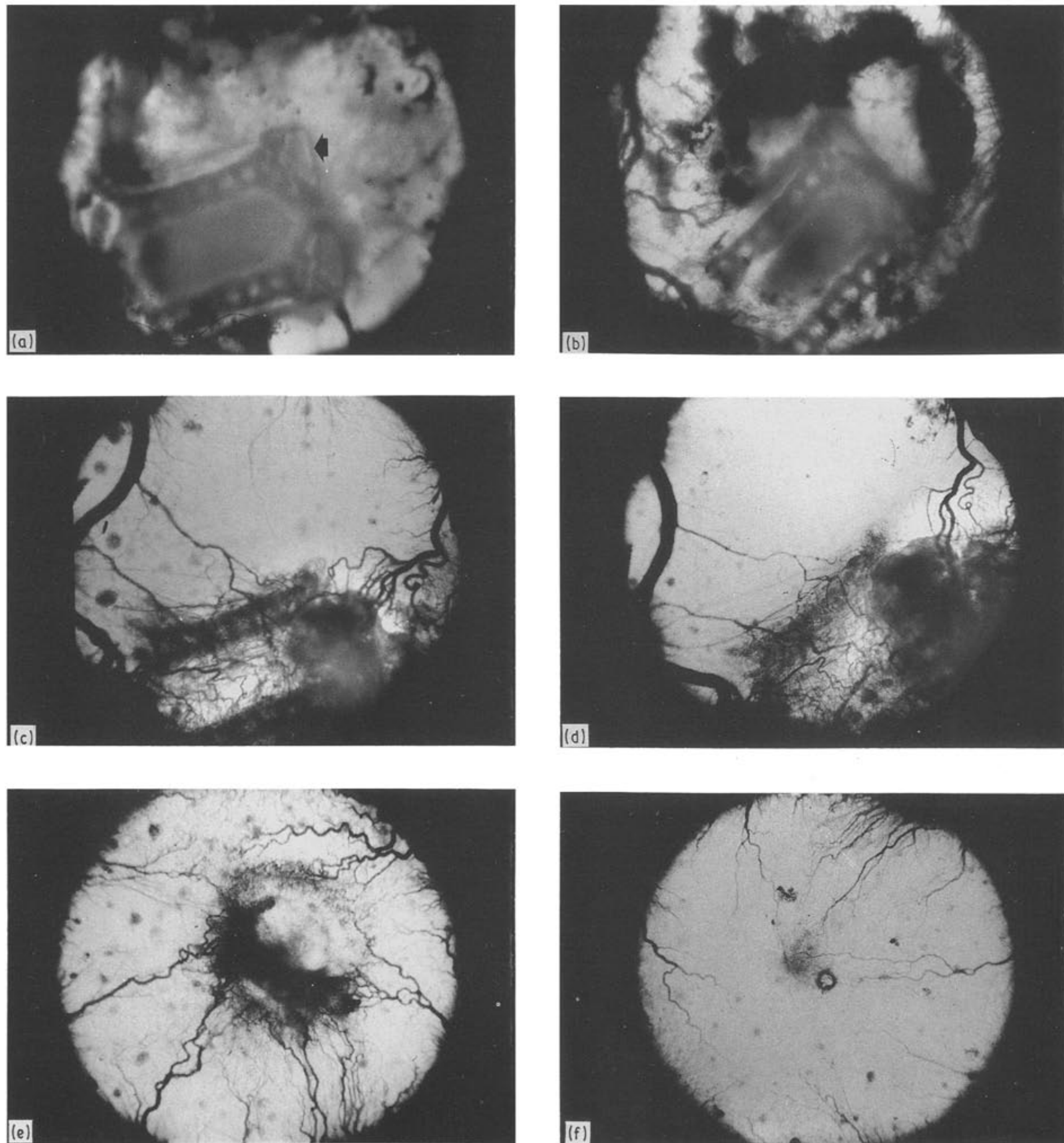
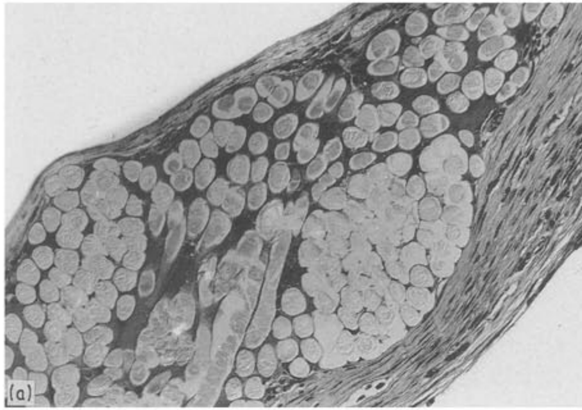


Figure 1 The *in vivo* breakdown of collagen–vicryl composite as observed *in situ* in the rabbit ear chamber. (a)–(d) show the destruction of EO-sterilized type 12 composite. (a) At 7 days after implantation, the implant is shrouded in inflammatory cells. The collagen associated with the vicryl mesh is still visible (arrow). (b) By day 14 the implant is beginning to clear of cells. Note the ingrowth of new blood vessels. Some haemorrhaging has occurred in the chamber. This sometimes occurs due to the rabbit knocking the chamber and is not associated with the implant. (c) By day 28, the vicryl mesh is surrounded by small blood vessels some of which appear to run over and within the mesh. (d) By day 35, the mesh is fading in outline, and there are still many small blood vessels associated with the implant. (e) The state of a piece of gamma-irradiated material (type 12) at 28 days. Note the fragmented appearance of the material and the shrouding of the implant with inflammatory cells in comparison with (c). (f) 35 days after implantation, the gamma-irradiated type 12 material has almost disappeared (original mag. × 12).

involves the removal of the collagen film followed by the dissolution of the vicryl component. The collagen-removal phase seems to require cellular mechanisms, presumably enzyme-mediated breakdown of the film followed by phagocytosis. By 17 days the collagen film was well infiltrated by the cells and by day 35 had totally disappeared. Implanted materials were quickly shrouded in inflammatory cells. It is known that newly formed blood vessels in the growing granulation tissue are "leaky" and allow easy migration of inflammatory cells into the tissue space [26]. Such cells are a major component of the leading edge of the growing tissue and are also present in the fluid-filled part of the chamber, having moved out from the advancing tissue front. Recruitment from both of these cell pools results in the "shrouding" of the implants. This effect was absent if the material was placed on an established bed of granulation tissue (which totally filled the chamber). In these cases details of the implant structure remained clearly visible throughout the period of *in vivo* observation and the histological results from such chambers at 35 days were identical to those detailed above [27]. Thus the shrouding of the implant by inflammatory cells was a consequence of its position in relation to the growing tissue front, rather than any adverse reaction to the material. It is probable that the



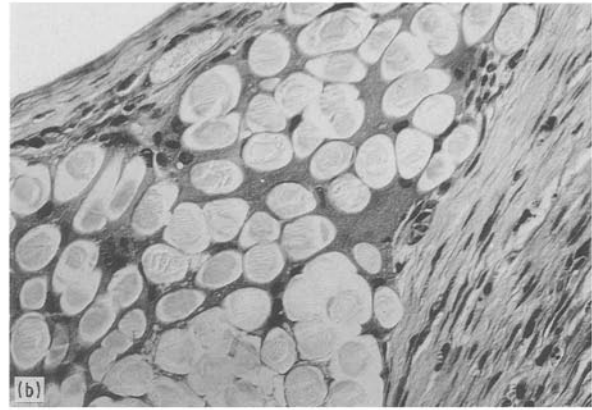


Figure 2 (a) 35 day section of ear chamber tissue showing type 9 vicryl (EO-sterilized) surrounded by granulation tissue. Note the low level of inflammatory cells associated with the fibres and the absence of the collagen film (H and E, × 138). (b) Enlarged detail of (a), showing inflammatory cells associated with the type 9 vicryl. Note also the deposition of collagen adjacent to some of the vicryl strands (H and E, × 355).

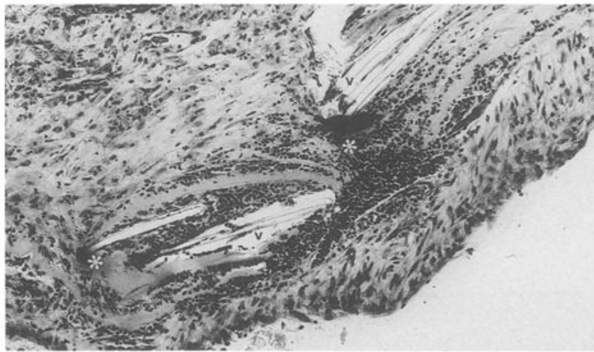


Figure 3 Collagen–vicryl type 12 composite 17 days after implantation. The collagen film (*) is detached from around most of the vicryl (v) and is heavily infiltrated with inflammatory cells (H and E, × 138).

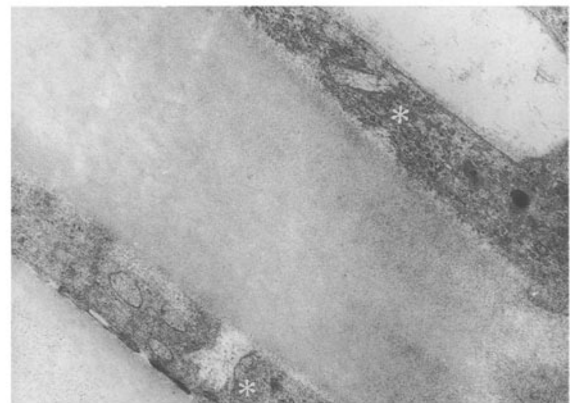


Figure 5 Electron micrograph showing cell pseudopodia extending along a strand of type 12 vicryl. Note the junctions between meeting pseudopodia (*). The original implant was sterilized with EO. (× 9788).

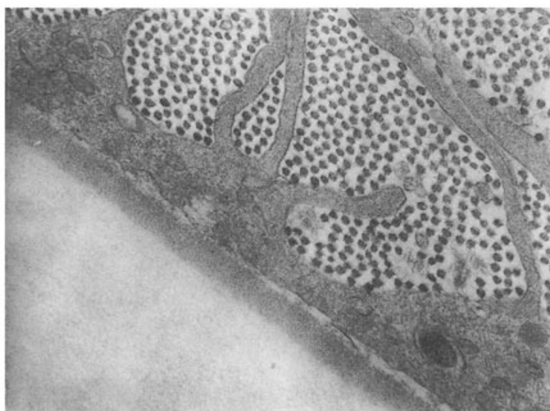


Figure 4 Electron micrograph showing a fibroblast attached to a type 12 vicryl strand, and engaged in the process of collagen fibrillogensis. Note osmophilic zone along the vicryl strand, presumably indicative of a hydration shell around the strand containing osmophilic cellular debris. The original implant was sterilized with EO (× 19 575).

"clearing" of the implant was related to the removal of the collagen film and that when it was eliminated the number of inflammatory cells associated with the remaining implant diminished.

At the end of our study, there were still substantial amounts of vicryl remaining from implants that had been sterilized with ethylene oxide or ethanol treated in contrast to those which had been gamma-irradiated. Previous studies have reported varying lengths of time taken for the total dissolution of vicryl. In one investigation the material had disappeared from histological sections at 6 weeks [22] whereas in another, virtually all vicryl remnants were absorbed only by 90 days [28]. A major difference between the two studies was the method of vicryl sterilization. The former used gamma-irradiated material whereas the latter used ethylene oxide-treated composites. In the light of our studies, this alone would explain the discrepancy in resorption times.

The breakdown of the composite involves the resorption of both collagen and the vicryl mesh. Whilst the resorption of implanted collagen in various forms by cells is well detailed in the literature, the cellular role in the breakdown of vicryl is still a matter of debate. In the current study no evidence of phagocytosis of, or lysosomal enzyme release on to vicryl was found. Vicryl is a copolymer of glycolic and lactic acids (containing 90% and 10% of each component, respectively) linked by esteric bonds and hydrolysis of these bonds is one of the most important, if not the only mechanism of polymer breakdown [28]. The

polymer is water soluble and has been shown to dissolve gradually in PBS and urine *in vitro* [29]. However, *in vivo* the cells associated with the implant contain a variety of enzymes which could potentially degrade the polymer, notably hydrolases and oxidases, although it is likely that enzyme activity would be limited to a thin surface layer of the material [30]. *In vitro* studies on the degradation of vicryl and other related polymers, have showed that enzymes (particularly esterase and carboxypeptidase), can have a significant effect on polymer degradation under some conditions [31–33]. However, a study of the absorption of polyglactin 910 both *in vivo* and *in vitro* showed that although the initial hydrolysis was independent of cellular enzyme activity, enzymes may be involved in the metabolism of the products of polymer degradation [34]. In another study phagocytosis of vicryl fragments by macrophages and polymorphonuclear leucocytes was detected at the end stage of polymer hydrolysis [35]. Thus, while enzyme-mediated hydrolysis is theoretically possible, it is unlikely to play a major role in the initial breakdown of the polymer. Rather, enzymes are involved in the cellular metabolism of polymer breakdown products and in the process of phagocytosis of the vicryl, during the end stages of resorption.

In vitro experiments have shown that hydrolysis of vicryl is pH dependent [29, 36], being more rapid at higher pH. Conditions favouring optimal hydrolysis are not likely to be found in the ear chamber, as the local pH is usually acidic [26]. In our study, fibroblasts and macrophages were the dominant cell types associated with the implant at 35 days. Whether the physical presence of the cells around the vicryl fibres has any bearing on the rate of hydrolysis of the latter is unknown. It is conceivable that the attached cells decrease the rate of degradation by excluding extracellular media from the hydration shell present around the resorbing vicryl fibres. Collagen fibrillogenesis in the vicinity of the vicryl fibres may indicate an attempt to isolate this inert, but resorbable material and could well be fuelled by the metabolism of vicryl hydrolytic products.

When the method of sterilization was the same, no difference in the resorption of the type 9 and type 12 vicryl meshes was observed up to 35 days. New collagen was laid down around the vicryl fibres but whether this (and its subsequent remodelling) would result in the formation of better quality scar tissue when one mesh type is compared with the other remains entirely a matter of speculation.

An interesting finding in the study is the effect of gamma irradiation on the breakdown of the composite. Whilst irradiation is known to minimize possible cytotoxic effects induced in materials after sterilization treatment, it appears to have affected both components of the prosthesis. It is known to alter the physico-chemical properties of collagen by causing cross-linking and protein chain scission which results in aggregation, fragmentation and changes in the configuration of the polypeptide chains [37, 38]. There is evidence that such treatment also weakens vicryl by initiating free radical chain scission reactions resulting in the destruction of part of the mesh at the sterilization stage [39]. Such an effect has already been described for another similar polymer Polyglycolic acid [31, 40, 41]. When such a material is then implanted, its breakdown is accelerated, due to the damaged components being more susceptible to degradation. This would explain the results seen above with irradiated materials. In contrast, ethylene oxide-sterilized and ethanol-treated materials did not exhibit accelerated resorption, suggesting that such sterilization procedures do not adversely affect the components. Indeed, there is no evidence that either ethanol or ethylene oxide treatment have any detrimental effect on either collagen or vicryl. Exactly what effect these treatments do have on the composite is an obvious area for further study.

In terms of surgical application of the materials, type 9 collagen–vicryl composite has been used in urological surgery on rabbits [20–22] and it was reported that the effectiveness of the repair was not compromised by the use of gamma-irradiated vicryl as opposed to ethanol-treated material [22]. However, it may be advantageous in some circumstances to have a material which is resorbed over a longer period of time. In the case of the vicryl component of the composite this can be achieved using polymer which has been sterilized using ethylene oxide and would last up to 90 days in tissue. The introduction of increased cross-linking into the collagen film would reduce its degradation rate. Indeed, traces of implanted chemically cross-linked collagen film have been identified histologically in healing tissue 90 days after surgery [42]. An increase in the persistence of the collagen film as an integral unit may be useful in some circumstances for controlling haemorrhage or fluid leakage and provide a longer lasting platform or template for cell growth across a wound.

In summary, the results show that the collagen–vicryl composites appear to be well tolerated and do not elicit any obvious adverse tissue reaction under the conditions and during the time scale of the study. The method of sterilization of the composite is critical in determining *in vivo* degradation rate of the material.

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