Secondary cytotoxicity of cross-linked dermal sheep collagens during repeated exposure to human fibroblasts

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We investigated commercially available dermal sheep collagen either cross-linked with hexamethylenediisocyanate, or cross-linked with glutaraldehyde. In previous *in vitro* studies we could discriminate primary, i.e. extractable, and secondary cytotoxicity, due to cell-biomaterial interactions, i.e. enzymatic actions. To develop dermal sheep collagen for clinical applications, we focused in this study on the release, e.g. elimination, of secondary cytotoxicity over time. We used the universal 7 d methylcellulose cell culture with human skin fibroblasts as a test system. Hexamethylenediisocyanate-cross-linked dermal sheep collagen and glutaraldehyde-cross-linked dermal sheep collagen were tested, with intervals of 6 d, over a culture period of 42 d. With hexamethylenediisocyanate-cross-linked dermal sheep collagen, cytotoxicity, i.e. cell growth inhibition and deviant cell morphology, was eliminated after 18 d of exposure. When testing glutaraldehyde-cross-linked dermal sheep collagen, the bulk of cytotoxic products was released after 6 d, but a continuous low secondary cytotoxicity was measured up to 42 d. As a control, non-cross-linked dermal-sheep collagen was tested over a period of 36 d, but no secondary cytotoxic effects were observed. The differences in release of secondary cytotoxicity between hexamethylenediisocyanate-cross-linked dermal sheep collagen, glutaraldehyde-cross-linked dermal sheep collagen and non-cross-linked dermal sheep collagen are explained from differences in cross-linking agents and cross-links obtained. We hypothesize that secondary cytotoxicity results from enzymatic release of pendant molecules from hexamethylene-diisocyanate-cross-linked dermal sheep collagen, e.g. formed after reaction of hydrolysis products of hexamethylenediisocyanate with dermal sheep collagen. Glutaraldehyde-cross-linked dermal sheep collagen contains residual cross-linking agents, which induce the bulk cytotoxicity. Apart from being sensitive to enzymatic degradation, glutaraldehyde-cross-linked dermal sheep collagen was also found to be sensitive to aqueous hydrolysis. Hydrolysis of cross-links may release cytotoxic products and introduce new pendant molecules within glutaraldehyde-cross-linked dermal sheep collagen, which in turn induce cytotoxicity after enzymatic attack.

Keywords: Collagen, cross-linking, fibroblasts, cytotoxicity

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Collagen-based materials intended for use *in vivo* may be cross-linked to increase their strength and persistence. However, remnants of cross-linking agents, aqueous hydrolysis of cross-links or released fragments of cross-linked collagen may induce cytotoxicity¹⁻⁷. Our group investigated commercially available cross-linked dermal sheep collagen (DSC). The general aim of our studies is to obtain detailed knowledge in order to develop DSCs for clinical applications.

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Previous *in vitro* studies discriminated between primary cytotoxicity, due to direct leakage of products from the materials and secondary cytotoxicity, due to release of cytotoxic products from cell-biomaterial interactions, i.e. enzymatic actions. This phenomenon was clearly observed with hexamethylenediisocyanate-cross-linked DSC (HDSC)^{8,9}, when continuous release of cytotoxic products was measured with extracted HDSC, whilst no cytotoxic products were found in their extracts. In case of glutaraldehyde-cross-linked DSC (GDSC), no clear distinction between primary and secondary cyto-

toxicity could be made. In contrast to HDSC, two mechanisms take effect with GDSC; besides being sensitive to enzymatic actions, GDSC was also found to be sensitive to aqueous hydrolysis⁹.

The aim of the present study was to focus on the secondary cytotoxicity, challenging cross-linked DSCs to human fibroblasts (HF) over a longer period of time. Over a longer period, because for *in vivo* applications it is important to know during which period cellular interactions will release secondary cytotoxic products from DSCs and influence normal tissue ingrowth.

We used the universal methylcellulose (MC) test system⁸⁻¹⁰. This *in vitro* model predicts possible cytotoxic and degradation effects of biomaterials *in vivo*, because both mechanisms, i.e. (limited) aqueous hydrolysis and cellular interactions, can occur in MC cell culture. Cellular interactions with HDSC, GDSC and with non-cross-linked DSC (NDSC) as a control, were tested for repeated 6 d periods (up to 42 d) by counting cell numbers and evaluating cell morphology *in situ* by phase-contrast light microscopy and electron microscopy.

MATERIALS AND METHODS

Materials

Human fibroblasts (established cell-line PK 84) were routinely cultured in RPMI 1640 medium (Gibco Biocult Co., Paisley, UK), supplemented with 10% fetal calf serum (FCS), 2 mM/ml glutamine (Glut) (Merck, Darmstadt, Germany), penicillin (Pen) and streptomycin (Strep), both 100 units/ml (Gibco). The cells were incubated at 37°C in air containing 5% CO₂. A stock solution of methylcellulose (MC), Methocel high viscosity (3000-4000 cps) from Fluka, Bio Chemica, Buchs, Switzerland, was prepared according to Iscove and Schreier¹¹, with Iscove's modification of Dulbecco's medium (IMDM) (ICN Biomedicals Inc., Costa Mesa, CA, USA) in a final concentration of 2.25%.

Dermal sheep collagen (DSC) was obtained from the Zuid Nederlandse Zeemlederfabriek, Oosterhout, The Netherlands. If non-cross-linked, the material is called NDSC (batch 409-018). Two types of cross-linked DSC were obtained: HDSC (batch 392-001), which was cross-linked with hexamethylenediisocyanate (HMDIC) and GDSC (batch 397-010), which was cross-linked with glutaraldehyde (GA). In short, HDSC was cross-linked for 15 h, at pH 7.5, with a HMDIC solution of 1.25% (Desmodur®) containing 0.3% of surfactant (Emulvin W®), both obtained from Bayer, Wuppertal, Germany. GDSC was cross-linked for 17.5 h with a 0.5% GA solution (Regulan GT 50® obtained from BASF, Basle, Switzerland), by slowly raising the pH from 5 to 7. Discs with a diameter of 8 mm were punched from HDSC, GDSC and NDSC. The individual weights were established and all discs were sterilized by γ -irradiation, 2.5 Mrad (Gammaster, Ede, The Netherlands).

Methods

Cultures

After washing twice with phosphate-buffered saline (PBS) (NPBI, Emmer-Compascuum, The Netherlands),

human skin fibroblasts (HF) were harvested from routine culture using 0.25% trypsin in Ca⁺⁺/Mg⁺⁺-free Hanks' Salt Solution (Gibco). The cells were centrifuged and resuspended in IMDM.

MC/HF mixtures, described in detail by van Luyn *et al.*⁸ were made by gently and thoroughly mixing HF/IMDM (30%) with MC (50%) and FCS (20%). Pen, Strep and Glut had been added to IMDM in order to obtain the same final concentration in the culture gel as described for the RPMI 1640 medium. A final volume of 4.0 ml of culture gel containing 5×10^4 HF⁸ was placed into each well of a six well tissue culture plate (Greiner, Alphen a/d Rijn, The Netherlands) using a plastic syringe. Such cultures were either used as control culture, or, if certain discs were placed on the culture gel, as test cultures.

All cultures were incubated at 37°C in air containing 5% CO₂. After 24 h, two discs of either HDSC, GDSC or NDSC, with a total weight varying between 30 and 35 mg, were put on top of the MC/HF mixture in each well (Figure 1). After 6 d, discs (called discs H6, G6 and N6, respectively) were removed from the cultures. For further cell-biomaterial interactions, these discs, first cleaned from remnants of culture gel with sterile gauze dressings, were placed on fresh cultures (again, 24 h after cell seeding) and incubated for a second period of 6 d (Figure 1). Discs from this second culture period are referred to as discs H12, G12 and N12, respectively. This culture procedure was repeated up to six times, resulting in discs H18, H24, H30, H36, H42, G18, G24, G30, G36, G42 and discs N18, N24, N30 and N36.

Cell counts

After every culture period discs and gel were removed. The cell layers remaining on the bottom of the wells were extensively washed three or four times with 4 ml of PBS to remove the gel completely. The cells were then trypsinized, resuspended and counted in a Bürker counting chamber.

The cell proliferation inhibition index (CPII), expressed as a percentage of cell proliferation in control culture, was calculated as follows:

$$\text{CPII (\%)} = 100\% - \left[\frac{\text{mean cell number of culture with discs}}{\text{mean cell number of control culture}} \times 100\% \right]$$

Cell numbers were calculated as the mean of counts $\pm$ s.d ($n = 3$).

Microscopy

In situ photography of the cells with a phase-contrast inverted light microscope was performed after washing when the cells were covered with PBS. For electron microscopic studies, the trypsinized cells, pooled from the three wells, were washed with PBS and centrifuged. The resulting pellets were fixed with 2% GA in 0.1 M PBS and cut into small pieces. Post-fixation was done in 1% OsO₄, 1.5% K₄Fe (CN)₆ in PBS and the cells were dehydrated in graded alcohols and embedded in Epon 812. Ultrathin sections were stained with uranylacetate, dissolved in methanol and examined with a Philips 201 transmission electron microscope, operated at 40 kV.

Figure 1 At $t = 0$, 4.0 ml of MC gel containing 5×10^4 HF was placed in each well of six well tissue culture plates. After 24 h, two discs of HDSC were placed on top of the gel and incubated for 6 d. **a**, Thereafter, cells were counted and discs were transported to a fresh 24 h HF culture. This was repeated up to 42 d cell culture, **b**.

RESULTS

Cell counts

HF were seeded from MC/HF mixtures at a density of 5×10^3 HF/cm². DSC discs were placed on the MC gel of cultures after 24 h (Figure 1).

Cultures with discs of HDSC showed an inhibition of cell proliferation of $51.5 \pm 5.3\%$ (called disc-H6, Figure 2a) after 6 d. To test further effects of cell-DSC interactions, these H6 discs were placed for a second period of 6 d on a fresh HF culture. After 12 d of exposure, a CPII of $34 \pm 1.2\%$ (disc-H12) was measured. The next culture period of 6 d resulted in a CPII of $29.2 \pm 3.8\%$ (disc-H18). However, a fourth to a seventh culture period of 6 d (cultures with discs-H24, -H30, -H36 and -H42) did not show significant inhibition of cell proliferation, compared to the control culture (Figure 2a).

Discs of GDSC tested in this culture system showed a CPII of $86.9 \pm 0.6\%$ (disc-G6, Figure 2b) after 6 d of culture. The second 6 d culture period resulted in a tremendous drop in cell-growth inhibition (CPII of $7.9 \pm 1.9\%$). Further challenging these discs, up to 42 d, to HF resulted in low CPIIs, which were not significantly different from each other in disc-G12, -G18, -G24, -G30, -G36, up to disc-G42 (Figure 2b).

When testing discs of NDSC, a CPII of $20.8 \pm 1.0\%$ (disc-N6, Figure 2c) was measured after the first culture period. During further exposures, in contrast to HDSC and GDSC, no significant inhibition of cell proliferation was measured, as compared to the control. (Figure 2c, discs-N12, -N18, -N24, -N30 and -N36.)

Microscopy

Macroscopic examinations showed cell-free zones (CFZ) upon disc removal in the cell layers of cultures with discs-H6, -G6 and also, although very small, with discs-H12, -H18 and discs-G12 through to -G42. Light microscopic examination of the control culture showed a multilayer of well-spread cells with a few vacuole-like and sometimes with small birefringent particles in the cytoplasm (as shown with disc-H24, Figure 3a). Cultures with discs-H6, -H12, -H18 and -G6, clearly showed decreased cell numbers, which is in agreement with the

Figure 2 Cell proliferation inhibition indices (CPII) (%), $\pm$ s.d.) of repeated culture periods. One day after cell seeding, DSC discs were repeatedly exposed to MC/HF for periods of 6 d (cell counting at day 7), **a**, cultures with discs of HDSC (discs-H6, -H12, -H18, -H24, -H30, -H36, -H42); **b**, cultures with discs of GDSC (discs-G6, -G12, -G18, -G24, -G30, -G36, -G42); **c**, cultures with discs of NDSC (discs-N6, -N12, -N18, -N24, -N30, -N36).

Figure 3 a, LM micrograph of a multilayer of completely spread HF cultured with disc-H24, 7 d after cell seeding. Some cells contain very small biorefringent particles (arrows). Original magnification $\times 720$. b, LM micrograph of HF cultured with discs-G6, 7 d after cell seeding. At the edge of the CFZ, decreased numbers with proportionally larger and poorly adhering cells with many biorefringent particles are found. Original magnification $\times 780$. c, LM micrograph of HF cultured with disc-G30, 7 d after cell seeding. Some cells poorly adhered and many cells contain biorefringent particles (arrows). Original magnification $\times 720$.

cell counts. Increased numbers of vacuole-like and biorefringent particles were observed in the cytoplasm of cells. This was more pronounced near the CFZ in cultures with discs-H6, -H12, -H18, but most extremely with disc-G6 (Figure 3b). Furthermore, proportionally larger, poorly adhering cells with elongated or spiderlike structures were observed at the edges of the CFZ.

Some of the cells of cultures with discs-G12 through to -G42 had adhered poorly and their cytoplasm contained several biorefringent particles (Figure 3c).

Although a non-confluent cell layer was observed with disc-N6, the cell morphology was similar to that of the control culture. Cell morphologies of cultures with discs-N12 through to -N36, and discs-H24 through to -H42 were also similar to the morphology of cells in the control culture (Figure 3a). Transmission electron microscopic (TEM) examination of trypsinized cells confirmed and extended the differences described above in cell morphology compared to the control culture. Previously, the biorefringent particles were found to represent lipid droplets^{8,9} (Figure 4a). In our study they were found in increasing numbers in cultures with discs-H6 through to -H18, and in very high quantities in cultures with disc-G6. These cultures, with increased quantities of lipid, showed a reduction in the amount and dilatation of rough endoplasmic reticulum (RER) and, most strikingly, many cytoplasmic inclusions with remnants of dead cells. Also enlarged cells were found in these cultures. Cells of cultures with discs-G12 through to -G42 sometimes showed an increase of vacuoles and dark inclusions (myeline bodies) in the cytoplasm.

Morphologies of cultures with discs-H24 through to -H42, and discs-N6 through to -N36 were more or less similar to the morphology of the control culture.

Constitution of the discs

During 42 d culturing the constitution of discs of HDSC (up to disc-H42) had not changed macroscopically.

The same was found for GDSC, although by TEM, in contrast with HDSC, very small detached fragments of collagen were observed in between cells of cultures with discs-G30 through to -G42 (Figure 4b). Also collagen fragments phagocytosed by HF were observed. Sometimes they were recognized from the presence of aluminium silicate crystals, which were, as previously described^{12,13}, present in DSC (Figure 4c).

Macroscopically, discs of NDSC became smaller and after the first culture period of 6 d, showed a rather smooth soap-like surface. By TEM, fragments of denatured DSC were found in between cells of cultures with discs-N12 through to -N36.

DISCUSSION

The aim of this study was to investigate secondary cytotoxicity, occurring as a result of interactions between (cross-linked) DSC and HF.

We used the MC cell culture previously described with HF as the test system⁸. Our results showed that:

1. Challenging HDSC to HF for three repeated 6 d culture periods (18 d), eliminates all cytotoxic substances.
2. Challenging GDSC to HF eliminated the bulk of cytotoxic products after 6 d, but a continuous low secondary cytotoxicity was measured up to 42 d.
3. Challenging NDSC for repeated culture periods to HF did not show release of secondary cytotoxicity.

Figure 4 **a**, TEM micrograph of trypsinized HF cultured with disc-H18 at day 7. Cells are in general larger and contain increased quantities of lipid droplets (L). More contracted or rounded cells, decreased presence of RER and cytoplasmic inclusions with remnants of dead cells (arrows) are observed. Original magnification $\times 7142$. **b**, TEM micrograph of trypsinized HF cultured with disc-G42 at day 7. Several contracted or rounded cells were observed, which contain small lipid droplets (arrows); the lipid has been (partly) removed during embedding procedure (a). Some detached collagen fragments from GDSC were observed between the cells (C). Original magnification $\times 7905$. **c**, TEM micrograph of trypsinized HF cultured with disc-G36 at day 7. One cell has phagocytosed a detached fragment of GDSC. The fragment is also recognized from the presence of aluminium silicate crystals (arrow). Original magnification $\times 10752$.

Challenging HDSC to HF eliminates all cytotoxic substances within 18 d of cell culture

As previously discussed^{8,9}, cross-links obtained with HMDIC are not sensitive to aqueous hydrolysis, or to enzymatic breakdown. This implies that the observed secondary cytotoxicity must result from enzymatic cleavage (e.g. by collagenase) within the collagen

molecule itself. We hypothesized that the release of pendant molecules, still coupled to collagen fragments¹⁴, is responsible for the induced secondary cytotoxicity. During cross-linking, pendant molecules may have formed from reaction of the first isocyanate group of HMDIC with an ϵ -amino group of DSC, whilst a terminal NH_2 group of the second isocyanate group was formed

Figure 5 Formation of pendant molecules in HDSC during cross-linking. Reaction of HMDIC with H_2O may form the hydrolysis products AICH and DAH. AICH may react with $\epsilon\text{-NH}_2$ groups of DSC and form a pendant molecule in HDSC. Furthermore, DAH may react with HMDIC, and this product may then react with DSC and form a longer pendant molecule in HDSC.

by aqueous hydrolysis¹⁴ (Figure 5). The same pendant molecule may be formed when, as side reaction, the HMDIC hydrolysis product 1-amino-6-isocyanatehexane (AICH) reacts with an ϵ -amino group of the collagen (Figure 5). As previously discussed^{8,9}, the hydrolysis product 1,6-diaminohexane (DAH) may be formed and induce primary cytotoxicity. Apart from this, DAH may react with HMDIC and form another, longer pendant molecule within the collagen network (Figure 5). Statistically, enzymatic attack of the collagen molecule, e.g. by collagenase, which cleaves between the amino acids glycine and leucine¹⁵⁻¹⁸, will at first release the pendant molecules (by cleavage at one or two sites, Figure 6a), inducing secondary cytotoxicity. After 18 d HDSC is purified from cytotoxicity (Figure 2a), but enzymatic attack, and thus degradation, continues, probably releasing intact cross-links (by cleavage at two to four sites, Figure 6a), still coupled to collagen fragments. If so, these products did not induce cytotoxicity.

This hypothesis was confirmed in a pilot study, in which we incubated non-cytotoxic discs-H24 with bacterial collagenase (Sigma, St Louis, MO, USA, E C 34243, 5 units/ml IMDM) for 24 h at 37°C (ratio 1 g:20 ml). We tested the cytotoxicity of the conditioned medium obtained and the treated disc-H24-c. In both cases, cell growth was not inhibited, and both macroscopically and by mechanical measurements clear degradation of disc-H24-c was found. Bacterial collagenase cleaves collagen at several sites¹⁹, so in this case degradation fragments with intact cross-links must have been released.

These results, as well as the early elimination of secondary cytotoxicity from HDSC, support the hypothesis of pendant molecules being responsible for secondary cytotoxicity.

a

b

Figure 6 **a**, Scheme of HDSC network, containing HMDIC cross-links and several pendant molecules. Possible enzymatic attack sites by which pendant molecules and intact cross-links can be released are shown. **b**, Scheme of GDSC network with GA cross-links and pendant molecules. Possible hydrolysis sites in GA cross-links ($\text{N}=\text{CH}$ groups, arrows), which may also introduce new pendant molecules in GDSC (p), are shown.

Challenging GDSC to HF eliminated the bulk of cytotoxic products after 6 d, but a continuous low secondary cytotoxicity was measured up to 42 d Previously, with GDSC, no clear distinction between primary and secondary cytotoxicity could be made, because several types of cross-links of GDSC are sensitive to aqueous hydrolysis⁹. We previously hypothesized that aqueous hydrolysis of GDSC in the MC gel culture is limited^{9,14}. This was confirmed in the present study, because disc-G42, in contrast to 40 d extracted GDSC⁹, had not changed into a gelatinous mass.

After releasing residuals of GA from GDSC, probably occurring within 6 d (Figure 2b), other cytotoxic products can be released after aqueous hydrolytic action⁷ at two sites within a cross-link, or at one site within a pendant molecule (Figure 6b). The latter action will further introduce pendant molecules in GDSC. Comparable to HDSC, pendant molecules can be released by enzymatic attack, and induce secondary cytotoxicity (Figure 6b). This model may explain the continuous release of cytotoxicity from GDSC, which will probably continue up to final degradation. This also explains the higher degree of degradation of GDSC compared to HDSC, observed by TEM as very small detached fragments of collagen with discs-G30 through to -G42 (Figure 4c).

Our findings of GDSC are in agreement with *in vitro* studies of other investigators^{7, 20-23}, who found low cytotoxic effects from GA-cross-linked collagens in time. Some of these authors^{21, 23} did not find confirmation for their *in vitro* findings *in vivo*. Other authors^{3, 22, 24} observed long-term *in vivo* cytotoxic effects from GA-cross-linked collagens, e.g. heart valves.

Low continuing *in vitro* cytotoxicity may either not be effective *in vivo*, or the choice of animal, application site, and, even more, the microscopical evaluation level may fail to demonstrate this response. In our *in vivo* studies^{12, 13, 25}, after subcutaneous implantations of pre-cultured GDSC-discs (disc-G6, Figure 2b), by use of TEM we found response for the observed low cytotoxicity of disc-G6 *in vitro*. With these discs, in contrast to the implanted non-toxic disc-H24, a low, but clear inflammatory reaction was found, after 10 d implantation²⁵.

Challenging NDSC for repetitive culture periods up to 36 d to HF did not show release of secondary cytotoxicity

We previously reported low release of extractable primary cytotoxicity, accompanied by sensitivity for aqueous hydrolysis of NDSC⁹. The present study, with repeated exposures, shows that cell-related degradation of NDSC also occurs at a very high rate. This is observed macroscopically as decrease in sizes of the discs, which also had very smooth, denatured surfaces, and microscopically as denatured collagen fragments amongst cells. The results of NDSC, with respect to the degradation rate and cytotoxicity, are in agreement with our *in vivo* findings^{12, 13, 25}. Moreover these results show, that secondary cytotoxicity is related to cross-linked DSC.

In conclusion, our series of fundamental studies^{8, 9, 14} and the present study on dermal sheep collagen *in vitro* may explain possible reactions of DSCs *in vivo*. This shows the advantages of the MC cell culture, especially when used during repeated culture periods, in mimicking and predicting the *in vivo* situation.

In vivo, primary cytotoxicity of HDSC, GDSC and NDSC can be avoided by controlled washing. In case of HDSC, the remaining secondary cytotoxicity will probably soon be eliminated by cellular interactions, not seriously influencing biocompatibility over time. After elimination of the secondary cytotoxic products from HDSC, a non-toxic biocompatible scaffold will be present. In the case of washed GDSC, only low cytotoxicity will be induced up to final degradation. Whether this is a disadvantage may depend on the application area and quantity of GDSC used.

The HDSC and GDSC available commercially are not biocompatible. For future experiments, our group will focus on modifications of non-toxic cross-linked DSC, e.g. cross-linking with acyl azide or carbodiimide²⁶.

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