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AN *IN VITRO* CYTOTOXICITY STUDY OF ALDEHYDE-TREATED PIG DERMAL COLLAGEN

A. COOKE*, R. F. OLIVER† AND M. EDWARD

From the Department of Biological Sciences, Dundee University, Dundee DD1 4HN

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Summary.—The cytotoxicity of aldehyde-treated collagen was assayed by measuring ^{3}H-thymidine incorporation in adult human skin fibroblasts grown in tissue culture for 1 or 3 days in the presence of pig dermal collagen cross-linked with formaldehyde or glutaraldehyde. A comparison was also made with collagen preparations washed for 2 weeks either at 15° throughout or partly at 15° and partly at 37°. Collagen treated with both formaldehyde and glutaraldehyde proved increasingly toxic with increase in the concentrations of aldehyde used. While the maximum toxic effect was observed after 1 day culture in formaldehyde-treated collagen, with thymidine uptake ranging from 4–48% of control values with 5–0·1% formaldehyde and a 15° wash, the toxic effect of glutaraldehyde treatment increased with longer exposure and at 3 days thymidine uptake ranged from 3–40% of control values with 0·05–0·001% glutaraldehyde and washing at 15°. Washing partly at 37° significantly reduced toxicity, the differences in thymidine uptake as compared with washing at 15° alone ranging from 34–50% with 1 and 0·3% formaldehyde respectively in 1 day cultures and from 14–37% with 0·02 and 0·005% glutaraldehyde in 3 day cultures. While fibroblasts actively grew and migrated when seeded on non-cross-linked collagen, only limited cell survival occurred on aldehyde-treated collagen.

FORMALDEHYDE, and particularly glutaraldehyde, are commonly used to control the physical and biological properties of a variety of collagen-based biomaterials such as heart valve and blood vessel prostheses, implantable collagen preparations and collagen dressings. It is well established that pre-treatment with aldehydes, which react with collagen to produce intramolecular and intermolecular cross-links, reduces the solubility, antigenicity and biodegradation of collagenous tissues (Woodroof, 1978; Chvapil, 1980; Oliver *et al.*, 1980; Oliver *et al.*, 1982*a*, *b*) as well as changing their mechanical properties (Van Noort *et al.*, 1982). Furthermore, experimental studies with fibrous dermal collagen allografts and heterografts have shown that such grafts become recellularized and revascularized when cross-linked with low concentrations of formaldehyde and glutaraldehyde (Oliver, Grant and Kent, 1972; Oliver *et al.*, 1976; Oliver *et al.*, 1980; Shakespeare and Griffiths, 1980). However, when increasingly higher concentrations of aldehydes are used there is an increasingly adverse peripheral cellular reaction and reduced recellularization of the grafts (Oliver *et al.*, 1976; Oliver *et al.*, 1980).

Since aldehydes are highly toxic to living cells it is most important to obtain a balance between the beneficial aspects of aldehyde treatment and toxicity which is most likely to yield successful therapeutic results. To examine this aspect of the preparation of transplantable collagen we describe here the use of a highly sensitive *in vitro* technique in which the cytotoxicity of aldehyde-treated collagen was assayed by measuring ^{3}H-thymidine up-take, and thus cell DNA synthesis, in adult human

* Present address: Department of Medical Genetics, Royal Hospital for Sick Children, Yorkhill, Glasgow.
† To whom all correspondence should be addressed.

skin fibroblasts grown in the presence of pig dermal collagen cross-linked with formaldehyde and glutaraldehyde.

MATERIALS AND METHODS

Preparation of dermal collagen.—Cell-free fibrous collagen was prepared by incubating sheets of pig dermis of 0·5–1 mm thickness in a solution of crystalline trypsin (Armour) for 4 weeks as previously described (Oliver *et al.*, 1980).

Cross-linked collagen was prepared by incubation (100 mg/ml) for 16 h at 15° on an orbital shaker with appropriate concentrations of glutaraldehyde (Sigma) or formaldehyde (BDH) diluted in phosphate buffer pH 7·2. After cross-linking the collagen was washed using numerous changes of sterile saline for 2 weeks either at 15° throughout or, midway through this procedure, washing while incubated at 37° for 4 days before returning to 15°.

Cell culture.—Normal adult human skin fibroblasts were subcultured in plastic flasks (Nunc) using Eagle's basal medium supplemented with 10% new-born calf serum (Gibco) and penicillin/streptomycin and fungizone (Flow). The cells were split on a 1:2 ratio and those used for experimental studies were in the passage range 9–20.

Cytotoxicity testing of collagen: incorporation of ^3H-thymidine into skin fibroblasts.—Pieces of collagen were cut to an approximate size of 1 cm^2, placed in 35 mm diameter plastic Petri dishes (Nunc) and 5×10^4 cells in 2 ml of culture medium added to each dish. Control dishes contained the same number of cells but without collagen. The dishes were incubated at 37° in sealed plastic boxes in a 95% air: 5% CO_2 atmosphere for 1 or 3 days. After incubation, fresh medium containing 1 μ Ci/ml of (methyl—^3H) thymidine, specific activity 45 Ci/mmol (Radiochemical Centre, Amersham) was added and the dishes incubated for 5 h before removing the pieces of collagen. The cells were rinsed once with phosphate buffered saline (PBS) containing 5 mM EDTA then removed in 0·5 ml PBS/EDTA and lysed with sodium lauryl sulphate (0·1% w/v) for 20 min at 60°. 50 μl aliquots of each lysate were spotted in duplicate on filter paper strips. These were washed for 15 min in cold 5% trichloracetic acid, then 70 and 100% ethanol, dried and counted on a Packard Tricarb liquid scintillation counter.

Cytotoxicity testing: growth of cells on collagen.—Pieces of collagen were cut to fit the bases of 35 mm diameter dishes. To study ^3H-thymidine incorporation into cells grown on non-cross-linked collagen 1×10^5 cells in 2 ml culture medium were added to each dish and incubated as described above. After 3 days fresh medium containing 2 μ Ci/ml ^3H-thymidine was added. Control dishes contained collagen and radioactive medium but without cells. After 4 h the medium was removed and the collagen rinsed in PBS/EDTA, cut into smaller pieces and transferred to Carnoy's fixative to remove unincorporated radioactive thymidine. The collagen was washed in ethanol, dried and counted on a scintillation counter.

For microscopic examination 3×10^5 cells were added to dishes containing non-cross-linked and aldehyde-treated collagen which had been washed at 37° and incubated for various times when the collagen was removed and processed for light and scanning electron microscopy.

RESULTS

Cell growth on non-cross-linked collagen

Starting with a sparse distribution of cells at day 1, the cells had formed a discontinuous monolayer with occasional migration of cells into the collagen matrix by the 5th day. More complete coverage was observed in older cultures, including the presence of mitotic figures, and there was deeper, but still sparse cell migration into the collagen. In a separate experiment using an initial seeding density of 1×10^4 cells per dish, cells survived on the collagen for at least 7 weeks when their

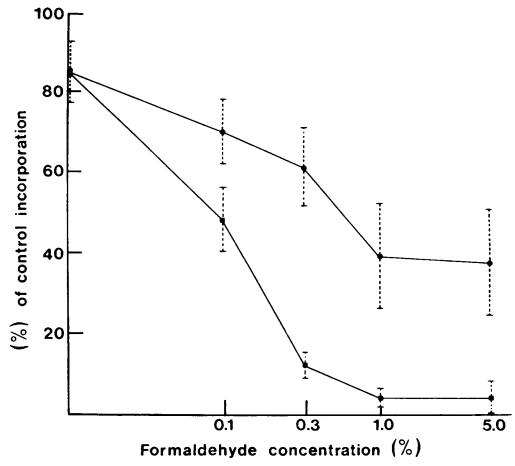

FIG. 1.—^3H-thymidine uptake by human fibroblasts after growing for 1 day *in vitro* in presence of formaldehyde-treated collagen expressed as % of control uptake (no collagen present); (■) collagen washed for 14 days at 15°; (●) collagen washed for 10 days at 15° and 4 days at 37°. Vertical bars represent 1 s.d. (n = 10).

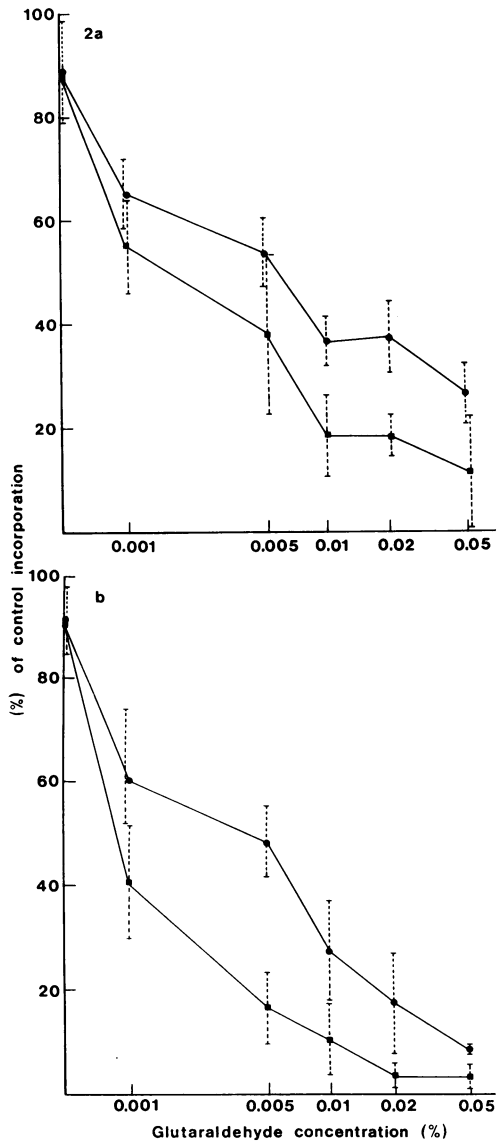

FIG. 2.—^{3}H-thymidine uptake by human fibroblasts after growing for 1 day (a) and 3 days (b) in presence of glutaraldehyde-treated collagen. Explanation as Fig. 1.

numbers increased by a factor of 5 between the 2nd and 7th weeks.

Confirmation of cell proliferation was provided by the ^{3}H-thymidine incorporation study. From 2 separate experiments the radioactivity in the preparations with cells was 1186 ± 191 dpm ($n = 6$) but only 50 ± 16 ($n = 7$) in the control collagen preparations without cells. Here it is interesting to note that while vigorous growth of fibroblasts also occurred on the plastic surfaces adjacent to non-cross-linked collagen, thymidine incorporation was reduced by some 10–15% when compared to cultures containing no collagen (Figs 1, 2a and 2b).

Toxicity of formaldehyde-treated collagen

Thymidine incorporation in fibroblasts was measured after 1 day (Fig. 1) and after 3 days in culture in the presence of collagen pre-treated with 0·1, 0·3, 1 and 5% formaldehyde and a comparison was made between collagen washed at 15° and material washed partly at 15° and partly at 37°.

The results showed that an exposure of only 24 h of fibroblasts to formaldehyde-treated collagen was required to produce the maximum toxic effect, there being little difference between the 1 day and 3 day culture results.

Formaldehyde-treated collagen washed only at 15° was extremely toxic to human fibroblasts. Thymidine incorporation in dishes containing collagen cross-linked with 0·1% formaldehyde was some 48% of the control level 1 day after seeding and 55% after 3 days. The corresponding values for 0·3% formaldehyde were 12% at 1 and 3 days and for 1% formaldehyde 4 and 2% respectively.

Significantly different results were obtained, however, with collagen which had been washed partly at 15° and for 4 days at 37°. The level of thymidine incorporation declined more gradually with increasing formaldehyde concentration, reaching 70% of the control value after 1 day culture and 75% after 3 days for cells growing in the presence of 0·1% formaldehyde-treated collagen. The corresponding values for 0·3% formaldehyde were 61 and 63% respectively and for 1% formaldehyde 38 and 41%. The differences in the extent of thymidine uptake attributable to the 37° washing procedure ranged from 34% with 1% formaldehyde to 50% with 0·3% formaldehyde.

Toxicity of glutaraldehyde-treated collagen

Experiments were carried out using identical procedures to the formaldehyde-treated collagen, but a lower concentration range of glutaraldehyde (0·001–0·05%) was employed.

The thymidine incorporation results with 1 day cultures (Fig. 2A) showed that, in common with the finding with formaldehyde, collagen washed for a period at 37° was less toxic to fibroblasts than preparations washed solely at 15°. However, the differences in thymidine uptake due to this washing regime were considerably lower, ranging from only 10% with 0·001% glutaraldehyde to 19% with 0·02% glutaraldehyde.

A similar trend was apparent with 3 day cultures (Fig. 2B), with collagen washed partly at 37° being less toxic to cell thymidine uptake as shown by increases ranging from 14% with 0·02% glutaraldehyde to 37% with 0·005% glutaraldehyde. However, unlike with formaldehyde, comparison of the thymidine incorporation levels found in cells exposed for 1 day and 3 days demonstrated that the toxic effect of glutaraldehyde increased with the longer exposure period. This was particularly noticeable with the lower concentrations of glutaraldehyde with collagen washed at only 15° and at higher concentrations with collagen washed partly at 37° where the difference in thymidine uptake ranged from 15–22% less than the 1 day culture levels of incorporation.

Despite the above results, sparse cell survival, as judged by light microscope examination, was observed on collagen pre-treated with both glutaraldehyde and formaldehyde and washed at 37°

DISCUSSION

The inherent acceptability of trypsin-prepared, non-cross-linked dermal collagen as a substrate for the *in vitro* growth of human fibroblasts was demonstrated both by the ^{3}H-thymidine incorporation results and the observed proliferation and migration of cells to cover the collagen surface. Nevertheless, an inhibitory effect on cell thymidine uptake (10—15%) occurred in fibroblasts grown adjacent to non-cross-linked collagen as compared to cultures containing no collagen.

Although limited cell survival appeared to occur on aldehyde-treated collagen, collagen cross-linked with both formaldehyde and glutaraldehyde proved increasingly more toxic, as judged by reduction in thymidine uptake, with increase in the concentrations of aldehyde used. While the maximum toxic effect was observed after 1 day culture with formaldehyde-treated collagen, the toxic effect of glutaraldehyde treatment increased with longer exposure. This toxic effect was presumably due to the leaching of unbound or loosely bound aldehydes from the collagen into the medium. However, toxicity was significantly reduced, especially following formaldehyde cross-linking, by including a 4 day wash at 37° in the 2 week washing procedure. In this context ^{14}C-labelled formaldehyde studies have shown that formaldehyde-collagen bonds are relatively unstable and that washing at 37° leads to rapid loss of formaldehyde from formaldehyde-treated pig dermal collagen (Barker, Oliver and Grant, 1980).

Woodroof (1978) states that the toxic effects of formaldehyde and glutaraldehyde on cells are apparent at concentrations greater than 10–20 parts/10^6 and recommended 3 separate 2 minute washes for tissue heart valves stored in 0·5% glutaraldehyde or 4% formaldehyde before clinical use. However, using extracts from glutaraldehyde cross-linked collagen sponge, Speer *et al.* (1980) found that concentrations of 3 parts/10^6 of glutarladehyde inhibited 3T3 fibroblast growth by 99% in tissue culture.

The *in vitro* findings correlated with results from implantation studies of aldehyde cross-linked dermal collagen (Oliver *et al.*, 1976; 1980) in which toxic effects were shown by an increased foreign body reaction and the persisting presence of multinuclear giant cells with higher concentrations of aldehydes. The foreign

body reaction observed with implants of glutaraldehyde cross-linked biomaterials has been attributed to the presence of residual unbound, water-insoluble polymers of glutaraldehyde (Chvapil, 1980; Speer *et al.*, 1980).

The results indicate that while preserving the beneficial aspects of aldehyde treatment of collagen biomaterials, particular care must be taken with cross-linking and washing procedures to minimize the short-term and possible long-term effects of aldehyde toxicity.

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