Table 4. Comparison of autoxidation rates (k_{obs} in h^{-1}) of molluscan and vertebrate myoglobins, at 25 °C in 0.1 M buffer

Sources	Reference	k_{obs}(pH 7.2)	k_{obs}(pH 8.8)	k_{obs}(pH 8.8)/k_{obs}(pH 7.2)
Sulculus	This work	0.030	0.59	19.7
Aplysia	16	0.158	0.100	0.63
Dolabella	12	0.044	0.048	1.09
Cerithidea	14	0.022	0.020	0.91
Sperm whale	18	0.010	0.0012	0.12

structure of the bound oxygen [17], it seemed that it would be of great interest to examine the stability of *Sulculus* myoglobin showing an unusual alpha/beta ratio.

Table 4 shows the first-order rate constant (k_{obs}) for the autoxidation of *Sulculus* oxymyoglobin at two different pHs, together with those of *Aplysia* [16], *Dolabella* [12], *Cerithidea* [14] and sperm whale [18] myoglobins. The autoxidation rate of *Sulculus* myoglobin at pH 7.2 was comparable to those of *Dolabella* (monomeric myoglobin) and *Cerithidea* (dimeric myoglobin), but at pH 8.8, where autoxidation is minimized in most of oxymyoglobins, the rate for *Sulculus* myoglobin was extremely accelerated. This can be clearly seen from the ratio of k_{obs}(pH 8.8)/k_{obs}(pH 7.2). As shown in table 4, the ratio for *Sulculus* myoglobin shows a higher value of 19.7, compared with the ratios (0.12–1.09) for the other myoglobins. Since autoxidation is understood to take place through a nucleophilic displacement mechanism by nucleophiles such as H_2O or OH^- [19, 20], this indicates that the heme pocket of *Sulculus* myoglobin might offer extremely easy access to the OH^- ion, resulting in very rapid autoxidation. These characteristics of autoxidation and also the spectral properties could be caused by structural distortion inherent in the unusual structure of *Sulculus* myoglobin.

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The growth of human fibroblasts and A431 epidermoid carcinoma cells on gamma-irradiated human amnion collagen substrata

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Summary. Human fibroblasts and A431 human epidermoid carcinoma cells were cultured on gamma-irradiated human amnion collagen as well as on plastic dishes and non-irradiated collagen coated dishes. The morphology, attachment, growth and short-term cytotoxicity of these culture conditions have been determined. Both irradiated and non-irradiated amnion collagen enhanced the attachment and proliferation of fibroblasts as compared to the plastic dishes. No differences in these properties were observed for A431 cells cultured on irradiated collagen when compared with culture on non-irradiated collagen substrates. Cytotoxicity assays showed that irradiated and non-irradiated collagens were not cytotoxic for either fibroblasts or A431 cells. The results demonstrated that amnion collagen irradiated at doses of 0.25–2.0 Mrads is optimal for cell growth.
Key words. Gamma irradiated collagen; injectable collagen; collagen substrata; gamma irradiation; amnion collagen; tissue culture.

Collagen, a family of fibrous proteins, is the major structural component of the vertebrate body [1]. Since it is immunologically benign, resistant to proteolysis, and a natural substrate for cell adhesion, collagen is an obvious choice for the manufacture of implantable prostheses [2, 3]. While reconstituted collagen has been employed for skin repair and replacement without cross-linking, most collagen materials are cross-linked to control their degradation and to further minimize antigenicity [4]. Since aldehydes are highly toxic [5], gamma irradiation has been used to sterilize and cross-link the collagen [6] preparations.

We have developed a new form of injectable human collagen to eliminate the xenogenic adversity of bovine collagen which is currently in widespread use for soft tissue augmentation [7, 8]. This injectable collagen was extracted from human amnion and irradiated by gamma rays for sterilization and cross-linking.

It has long been appreciated that collagen substrates are involved as agents in promoting cell attachment, cell shape and proliferation. The effect of gamma irradiated soluble amnion collagen on the growth of tissue culture cells has not been previously described. In this research, we compare normal fibroblasts and an epidermoid carcinoma cell line (A431) cultured on gamma irradiated injectable amnion collagen films so as to determine the optimal irradiation dosage for cell growth.

Materials and methods

Preparation of irradiated human amnion collagen film. Collagen was extracted from fresh human amnion by trypsin digestion and purified by selective precipitation from neutral salt and acid solutions [9]. The ratio of type I and type III collagens in these preparations is 44:56. Purified collagen solution at 1.5 mg/ml in 0.5 M acetic acid was irradiated in a 1779-Ci gamma rays source at a dose rate of 1000 rads/min. The dose level was varied over the range of 0.25–2 Mrads. Irradiation was carried out at room temperature; no gelation of the collagen solution occurred under these conditions.

The surface of culture dishes was coated with 1 ml of the collagen solution, and the excess liquid removed with a Pasteur pipette. The collagen films were then air-dried and washed 3 times with Hanks' balanced salt solution (HBSS) before use. Control dishes were treated with 0.5 M acetic acid or non-irradiated collagen solutions using the same procedure.

Cell culture. Normal baby human foreskin fibroblast cells were a gift from Dr. Malachy Gleason, Department of Urology, Baylor College of Medicine, Houston. Cells were propagated and confluent monolayers were serially subcultured by trypsinization. Cells between the third and sixth culture passage were used in these experiments. The standard culture conditions involved the use of Dulbecco's modified Eagle's medium (DME) in an air/CO_2 (95%:5%) atmosphere at 37 °C. DME was supplemented with penicillin (400 units/ml), streptomycin (50 µg/ml) and 10% fetal calf serum. The medium was changed three times per week.

The human epidermoid A431 carcinoma cell line (ATCC, Rockville, Maryland) was grown as described above in DME containing 5% fetal calf serum.

Cell attachment assay and cell counting. 5×10^4 per well of normal fibroblasts (or A431 cells) were incubated at 37 °C for 10, 20, 40, 60 and 90 min on 24-well plates. Unattached cells were removed by decanting the culture medium and washing three times with HBSS. The attached cells were released from the culture surface by treatment with 1 ml of Type II collagenase (Sigma, St. Louis, MO) solution (1 mg/ml) for 10 min and 3 ml of 0.05% trypsin in 0.53 mM EDTA (Gibco, Grand Island, NY) for another 10 min at 37 °C. The numbers of attached, unattached and total number of cells in these suspensions were determined using a Coulter counter.

^{3}H-thymidine incorporation. Normal fibroblast (5×10^4 cells) or A431 cells (1×10^4 cells) were seeded on 60-mm plastic petri dishes or collagen films. After incubation for 6 or 9 days, 2 ml of fresh medium containing 0.4 µCi/ml ^{3}H-thymidine (methyl-^{3}H-thymidine, sp. act. 84.8 Ci/mmol NEN, DuPont) was added and the plates were incubated for 30 min. The cells were rinsed twice with cold phosphate buffed saline and scraped off. After centrifugation, the cell pellets were sonicated twice in 3 ml cold 10% trichlorocetic acid and centrifuged. The pellets were resuspended in 0.5 ml of 0.5 M peracetic acid and sonicated, the sonicates were then heated at 70 °C for 45 min and centrifuged. 400 µl of the supernatant fractions were then neutralized with 100 µl of 2 N NaOH and counted in a Beckman LS7800 liquid scintillation counter.

Short-term viability test. Cell suspensions were prepared by trypsinization after incubation for 8 days. Short-term viability tests were performed by counting the stained cell number and the total cell number after staining with trypan blue. Viability was calculated by determining the ratio of dead cells (stained) to total cell number.

Data analysis. Standard statistical methods were used. T-tests were used to determine differences between the means of 2 groups examined.

Results

Morphology. Fibroblasts growing on plastic dishes, or non-irradiated, or irradiated collagen films eventually form a contact-inhibited monolayer which exhibited a typical appearance at confluent cell densities. There were no morphological differences among the different cell populations tested. Freshly seeded fibroblasts were round. Once the initial sequence of attachment and spreading was complete, the cells appeared homogenous

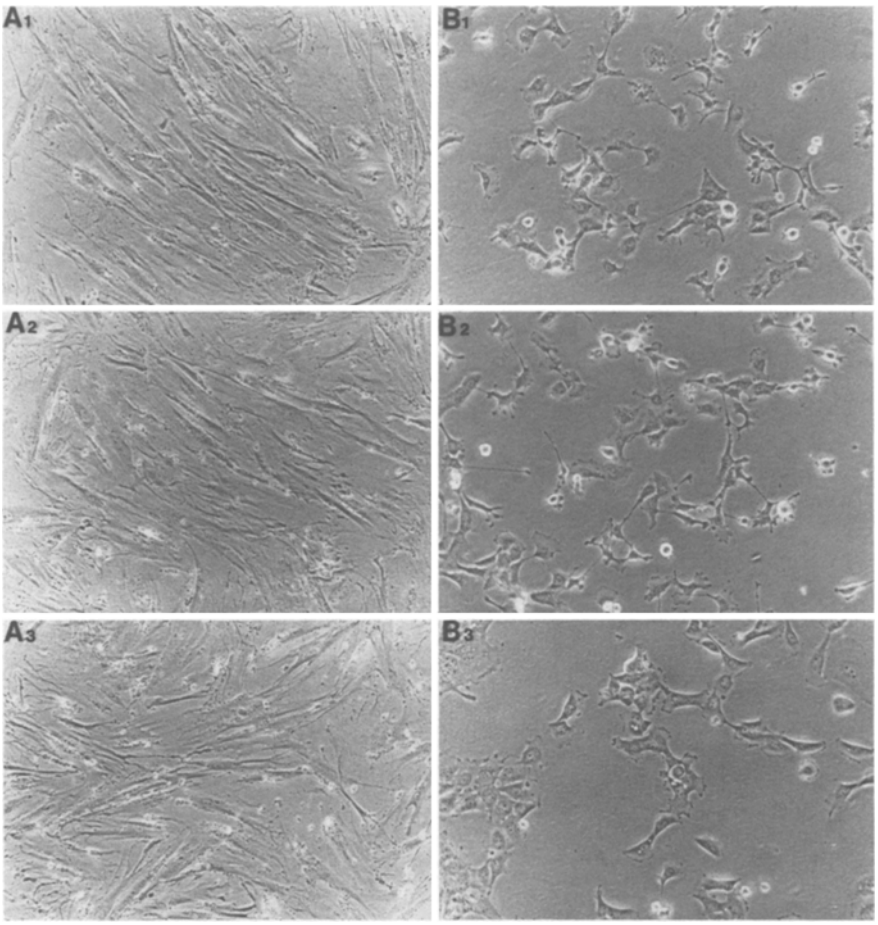

Figure 1. The appearance of normal fibroblast (A1–A3) and A431 cells (B1–B3) growing on plastic dishes (A1 and B1) non-irradiated (A2 and B2) and 2 Mrads irradiated (A3 and B3) collagen coated dishes. Fibroblasts and A431 cells were photographed at 8 days or 3 days after initial plating respectively. (A1–A3, × 125; B1–B3 × 250).

exhibiting the characteristic polarized-shape morphology of the normal fibroblast (fig. 1).

The A431 cells growing on irradiated collagen films showed no morphological differences from the cells cultured on non-irradiated collagen films nor from the cells cultured on plastic dishes. Active needle-like microspike and sheet-like lamellipodia projected outward from the typical colonies.

Cell attachment. The kinetics of fibroblast attachment to plastic dishes and to irradiated and non-irradiated collagen-coated dishes was studied. The data are expressed as the percentage of the total number of cells adherent on the substratum (fig. 2).

As can be seen in figure 2, at 20-min and 40-min incubation periods, fewer normal fibroblasts adhere to the plastic dishes than to amnion collagen-coated dishes. The attachment of cells to the irradiated collagen films varied with the irradiation dosage of the amnion collagen. Cell attachment to 0.25 Mrads irradiated collagen-coated dishes occurred to the same extent as non-irradiated col-

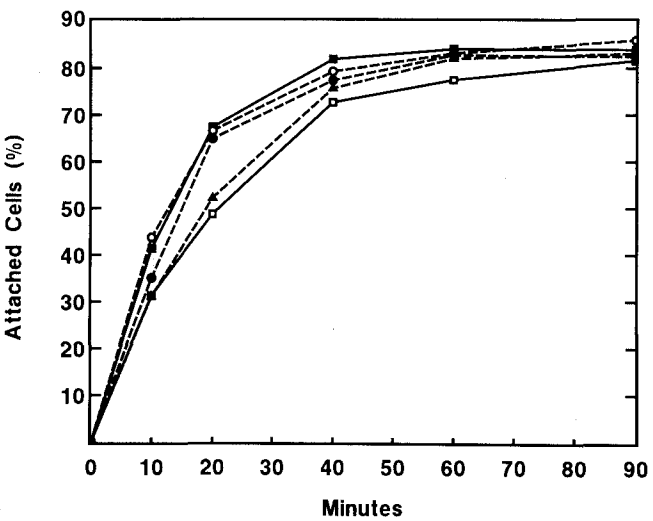

Figure 2. The kinetics of fibroblast attachment to various substrata. A total of 5×10^4 cells was initially plated on each dish. Cell attachment to plastic dishes (□) non-irradiated (■) or 0.25 Mrads (○), 1 Mrads (●), 2 Mrads (▲) irradiated collagen-coated dishes are expressed as the percentages of the total cells adherent to the substrata.

lagen-coated dishes. As the irradiation dosage increased to 2 Mrads, fibroblast attachment decreased. Although the cells adhered more rapidly to non-irradiated collagen-coated dishes, a plateau value was eventually reached for all cell cultures. After 60 min, more than 80% of the cells had attached to the substrata.

In contrast, gamma-irradiated collagen did not affect cell attachment of A431 cells at all time periods of incubation. (Data not shown.)

In each case, cells were observed microscopically; they were morphologically intact and able to exclude trypan blue.

Cell proliferation. Cell growth on various substrates was measured by counting cell numbers at days 3, 6, 9 after incubation, and by ^{3}H-thymidine incorporation at days 6 and 9 post incubation.

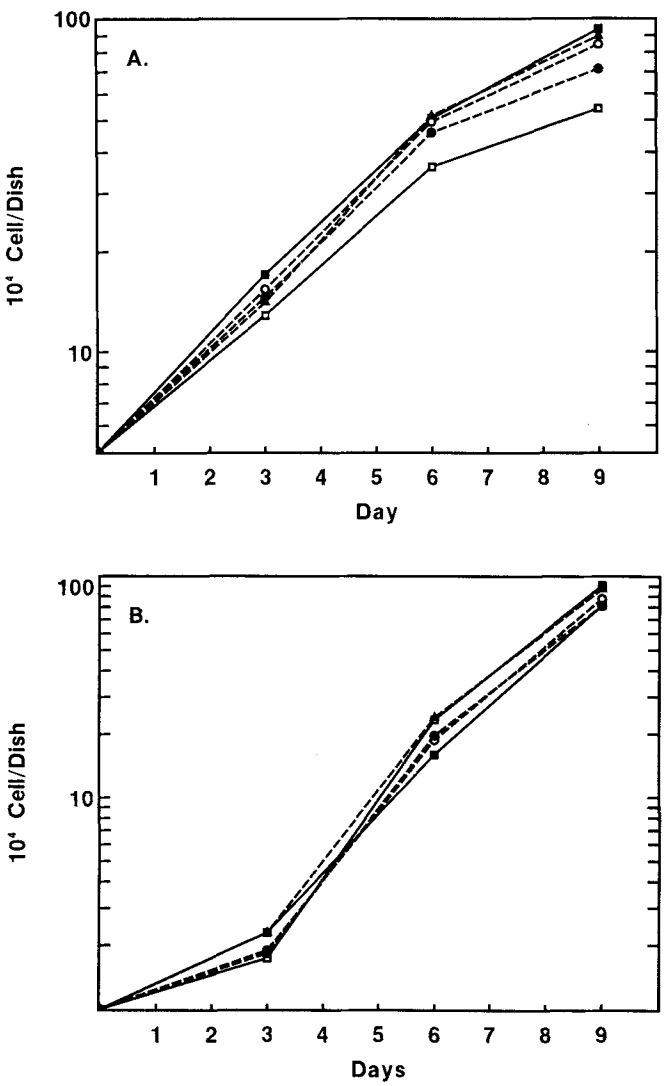

Figure 3. The growth of normal fibroblasts (A) and A431 cells (B) on plastic dishes (□) and non-irradiated (■) and 0.25 Mrads (○), 1 Mrads (●) and 2 Mrads (▲) irradiated collagen-coated dishes. Each point in the figure is the mean of quadruplicate determinations and the SD was always less than 10% (not shown).

The growth characteristics of normal skin fibroblast and A431 cells on plastic dishes and on irradiated and non-irradiated collagen-coated dishes are shown in figures 3 A and 3 B, respectively.

The growth characteristics of normal skin fibroblasts showed some differences on the various substrata. Growth on the non-irradiated and irradiated (0.25 Mrads, 1.0 Mrads and 2.0 Mrads) collagen film occurs at approximately the same rate, while the fibroblasts cultured on the plastic dishes grow at a significantly slower rate ($p < 0.05$) (fig. 3 A).

Confirmation of these differences in growth rate was provided by the ^{3}H-thymidine incorporation study. ^{3}H-thymidine incorporation for fibroblasts on plastic dishes is only 75.6% and 61.2% of that of fibroblasts on non-irradiated collagen-coated dishes on day 6 and on day 9, respectively. No significant differences in ^{3}H-thymidine incorporation were observed among cells growing on irradiated collagen and non-irradiated collagen films. These results demonstrate that gamma-irradiated collagen exhibits the same biocompatibility as the non-irradiated collagen with respect to cell proliferation (table).

^{3}H-thymidine incorporation by fibroblasts grown on plastic, non-irradiated and irradiated collagen substrates

Substrate	Day 6 ($\times 10^5$ dpm)	Day 9 ($\times 10^5$ dpm)
Plastic	1.21 ± 0.12*	1.91 ± 0.14*
Non-irradiated collagen	1.60 ± 0.15	3.12 ± 0.29
0.25 Mrads irradiated collagen	1.67 ± 0.13	2.73 ± 0.21
1 Mrads irradiated collagen	1.61 ± 0.14	3.00 ± 0.29
2 Mrads irradiated collagen	1.73 ± 0.09	3.10 ± 0.19

*$p < 0.05$ for comparison between plastic and collagen-coated dishes.

The growth characteristics of A431 cells on plastic dishes and on non-irradiated collagen-coated and irradiated collagen-coated dishes are shown in figure 3 B. The proliferation rates and saturation densities of A431 cells were the same on all of the substrata. ^{3}H-thymidine incorporation was consistent with the results of the cell growth assay (data not shown).

After 8 days' culture the cytotoxicity of collagen was assayed by trypan blue staining. No significant differences were found in the viability of normal fibroblasts or A431 cells growing on plastic dishes, irradiated collagen coated dishes and non-irradiated collagen coated dishes ($p > 0.05$).

Discussion

For soft tissue augmentation, injectable collagen is implanted intradermally in the papillary dermis, which contains fibroblasts and epithelial cells[11]. Host fibroblast ingrowth into the injected collagen occurs several days after implantation[12]. To assess the suitability of gamma-irradiated collagen for implantation, fibroblast and epithelial cell culture systems were employed.

The present study showed that injectable amnion collagen promotes fibroblast attachment. The attachment rates for fibroblasts growing on collagen-coated dishes were higher than those growing on plastic dishes. No significant adverse effect from irradiated collagen was noted at irradiation dosages of 0.25 M and 1 Mrads. When the irradiation dose increased to 2 Mrads, cell attachment rates were slower at 10, 20, and 40 min, but not lower than those on plastic dishes. At these high doses of irradiation (2 Mrads), cell attachment eventually reached the same level as that of cells growing on non-irradiated collagen-coated dishes. In contrast, with a tumor cell line (A431), attachment to the different substrates occurs at the same rate.

Further analysis has been presented concerning the proliferation of fibroblasts and A431 cells on plastic dishes, and non-irradiated and irradiated amnion collagen films. A431 cells were found to proliferate at approximately the same rate on all substrata, while human skin fibroblast cultured on collagen films (both irradiated and non-irradiated) grew faster than those cultured on non-collagen plastic dishes. No significant differences were noted for fibroblasts growing on the non-irradiated and various doses irradiated collagen films.

Thus, while normal fibroblast attachment and proliferation are promoted by collagen, no obvious effects were found on the attachment and growth of A431 cells. These results are consistent with the study by Klebe[12] and Liotta et al.[13]. Most untransformed mammalian cells require an appropriate surface for survival and growth in vitro, but not for the growth of their tumorigenic counterparts.

The cytoxicity study showed no obvious toxic effect of gamma-irradiated collagen on fibroblast and A431 cell growth. Furthermore, gamma-irradiated amnion collagen did not induce any observed morphological changes in cultured cells.

In light of these observations, we suggest that gamma-irradiated amnion collagen does not exhibit any adverse or toxic effects on fibroblasts and A431 cells. It allowed normal cell attachment, survival and growth. Monolayer cells cultured on gamma-irradiated and non-irradiated collagen eventually reached confluence at approximately the same time. We therefore suggest that low-dose gamma-irradiated collagen may prove to be an important biomaterial for plastic surgery.

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(*R*)-(*Z*,*E*)-9,11-octadecadien-13-olide: An intriguing lactone from *Heliconius pachinus* (Lepidoptera)

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Summary. A fourteen-membered lactone, (*R*)-(*Z*,*E*)-9,11-octadecadien-13-olide, was isolated from extruded abdominal glands of a Neotropical, nymphalid butterfly, *Heliconius pachinus* (Lepidoptera). This compound was obtained from mature adults of both sexes, but was not detected in young adults or pupae.
Key words. Butterfly; neotropical; *Heliconius pachinus*; fourteen-membered lactone.

Pheromones are chemical compounds, secreted by an individual, which influence the behavior or development of other individuals of the same species[2]. Many pheromones have been identified, particularly from insects. These include sex attractants, aphrodisiacs, alarm signals, trail markers, and substances with a variety of other activities[3]. An initial search for a new pheromone is usually based on a behavioral or electrophysiological