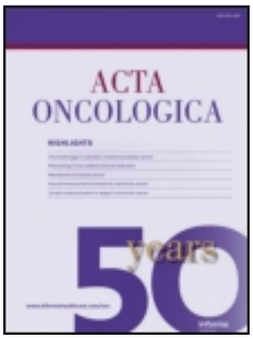

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Extracellular Matrix (ECM) and Cytoskeletal Modulation of Cellular Radiosensitivity

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Modulation of radiosensitivity by components of the extracellular matrix (ECM) and cytoskeletal elements has not been adequately studied. Although differences in the radiosensitivities of cells grown as monolayers, as spheroids, or grown in vitro in animal models are known, explanations have in the past neglected possible influences by the ECM and cytoskeleton. Using collagen gel cultures, it is shown that the fibrillar component of the ECM (which is responsible for cell anchorage) induces shifts in radiosensitivity. The effect is critically dependent on the affinity of the cell type towards collagen. The shifts in radiosensitivity induced by ECM alteration are manifested as changed D_q values. By applying four specific cytoskeletal poisons which either stabilize or destabilize specific cytoskeletal elements, the involvement of microfilaments and microtubuli was qualitatively appraised. Cytochalasin B, which destabilizes microfilaments (by preventing polymerization), caused a significant rise in radioresistance. This rise was due to increased D_0. Although the cellular morphological change accompanying cytochalasin B treatment was essentially similar to that obtained with trypsin, the respective shifts in radioresponses were qualitatively different and opposite, suggesting differences in mechanism of action.

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Discrepancies in the radiosensitivities of cells grown in vitro as monolayers, spheroids or grown in vivo (as solid tumours) have been well documented (1–5). Among the explanations offered are contact effect (6, 7) arising from cell–cell interactions, oxygen effects (8, 9) including charge potentials of cell surfaces (10) and cellular environmental factors. This last factor, concerning the cellular microenvironment, has been lucidly discussed by R. E. Durand (11). However, that review is confined to physico-chemical factors such as O_2 and pH in conjunction with blood flow through tumours. The cellular microenvironment is composed of more constituents than just solutes and fluids within the intercellular spaces. The extracellular matrix (ECM) which occupies these spaces is intricately structured and innately involved in regulatory processes ranging from the control of cell proliferation to tissue differentiation and, hence, ultimately the expression of gene functions.

The ECM consists of certain ubiquitous macromolecules such as collagen together with more tissue-specific ones such as laminin, elastin, fibronection (and an array of other 'nectins') which provide for cell anchorage. It further consists of various proteoglycans and a growing list of other non-collagenous proteins (e.g. nidogen, thrombospondin, etc.) which occupy sites both within the cell glycocalyx and the interstitial macromolecular lattice. These mediate and modulate regulatory stimuli. Appropriate cellular reactions to noxal stress including radiation insult depend on the integrity of the ECM, which is determined, in turn, by interactions among the cells themselves and with the matrix.

Besides mechanical sustenance, the cytoskeletal elements of the cell are probably responsible for vital mediatory events occurring between the ECM and the cell nucleus. Conceivable cytoskeletal influences on radiation response have not been investigated. Three structurally distinct principal elements constitute the cytoskeleton: microfilaments, microtubuli and intermediate filaments. The intermediate filaments may show molecular specificities for different cell types depending on their histological origins (see 1). Under normal physiological conditions the molecular constituents of the cytoskeletal elements exist in dynamic equilibrium with opposing degradative and synthetic events. Using definite cytoskeletal poisons, it is possible specifically to block either of the two processes (see 1). Such substances are available for microtubuli and microfilaments, respectively. Within a certain concentration range, withdrawal of the respective substance reverts the process without discernible negative effects.

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In this study, the influence of the principal fibrillar component of the ECM on the radiosensitivity (in terms of clonogenicity) of human diploid fibrobalsts (HDF) and V79 cells (transformed Chinese hamster cells) was assayed after x-irradiation. As an attempt to analyse the mechanism of action, trypsin treatment (which destroys the ECM) was applied. Furthermore, cytoskeletal poisons were used in order to understand possible involvement in radiation response of two different components of the cytoskeleton—microfilaments and microtubuli.

MATERIAL AND METHODS

Cell cultures

Two cell types were used in this study. 1) a Phase II human diploid fibroblast (HDF) strain IMR-90, which is not immortalized, 2) the other cell type was V79 which is a transformed cell line of hamster fibroblast origin. As details of culture conditions and growth characteristics have been described in earlier communications (12, 13), only specific aspects of the methodology applied in the present experiments are given here.

Monolayer cultures were maintained under standard conditions, i.e. Eagle's Minimum Essential Medium (MEM) supplemented with 15% foetal calf serum (FCS); 5% CO_2 in air at saturated humidity; thickness of medium layer = 0.2 ml/cm^2; no antibiotics. Collagen gel cultures were prepared with the same medium with the exception that the medium was semi-solid owing to polymerization of the collagen, the cells being entrapped within the polymerizing fibrillar mesh. This was achieved by mixing a calculated number of cells in FCS together with the medium before polymerization began. The medium itself consisted of seven parts collagen solution (2 g/l in 0.1% acetic acid prepared from rat tail tendon (Serva, Heidelberg, Germany)) mixed with one part 10× concentrated MEM and two parts $NaHCO_3$ (11.76 g/l in H_2O) at ice-bath temperature to check polymerization. After adding the cells in FCS, aliquots were dispensed into culture dishes and the temperature quickly raised to 37°C by placing the dishes on a heated plate. Polymerization occurred within 10 min.

Partial ECM dissolution with trypsin

Partial ECM destruction was achieved by treatment with a 0.25% trypsin solution without EDTA. The cultures were rinsed once with cold trypsin and then covered with 0.5 ml/25 cm^2 allowing exposure for about 4 min. This treatment caused retraction of cytoplasmic processes and a corresponding rounding of cells because of disruption of cell anchorage. The enzymatic process was stopped before the cells became detached, by adding 5 ml culture medium containing 15% FCS. The cells were then irradiated.

Cytoskeletal poisons

The effects of four specific cytoskeletal poisons, viz. cytochalasin B, phalloidin, nocodazole and taxol (Sigma, St. Louis, MO, USA), were investigated. In a preliminary series of cytotoxic tests based on microscopic observation of cytopathological effects, each substance was checked for time and concentration effects by treatment of interphase V79 cells in exponentially growing monolayer cultures. The reversibility of effects upon withdrawal of the respective substances by replacement of medium was also recorded for each substance. The concentration and duration of treatment for each substance had to be selected such that, after withdrawal of treatment, no negative effect on clonogenic growth was observable. The substances were initially dissolved in dimethylsulphoxide (DMSO). Subsequent dilutions were in culture medium.

Cytochalasin B and phalloidin

The cytological effects of cytochalasin B and phalloidin were tested at concentrations ranging from 0.5 to 10 mg/ml. For the experiments in this study both substances were used at 10 mg/ml. At this concentration cytochalasin B induced cytoplasmic retraction and rounding of the cell within 10 min. Withdrawal of cytochalasin reversed the process within 10–15 min. Phalloidin did not induce any morphological change.

Nocodazole and taxol

Nocodazole and taxol were also tested for cytological effects. The concentrations tested ranged from 0.01 to 5 mg/ml. At a concentration of 1 mg/ml or higher, immediate cytotoxic effects were visible within 10–15 min. The cytotoxic effects were manifested as cytoplasmic 'pinching' and blebbing. At 5 mg/ml, the cultures degenerated, manifesting extensive karyorrhexis. The concentration chosen for both substances was 0.2 mg/ml. Mitotic block was recorded for all concentrations tested.

Irradiation of cell cultures

Cells were irradiated using a Philips RT 250 therapeutic x-ray machine which was operated at 250 kVp and 15 mA with a 2 mm built-in Al-filter. The HVL was 0.39 mm Cu. Dosimetry was checked with a Victoreen dosimeter and by LiF thermoluminescence dosimetry (TLD). With provision only for partial back scatter, the dose-rate was 2.2 Gy/min. Irradiation was carried out at room temperature. Culture flasks were removed from the incubators and placed under the x-ray source without delay.

Clonogenicity assays

The clonogenicity of the irradiated cells was assayed by colony scores. Cells which were dispersed using standard

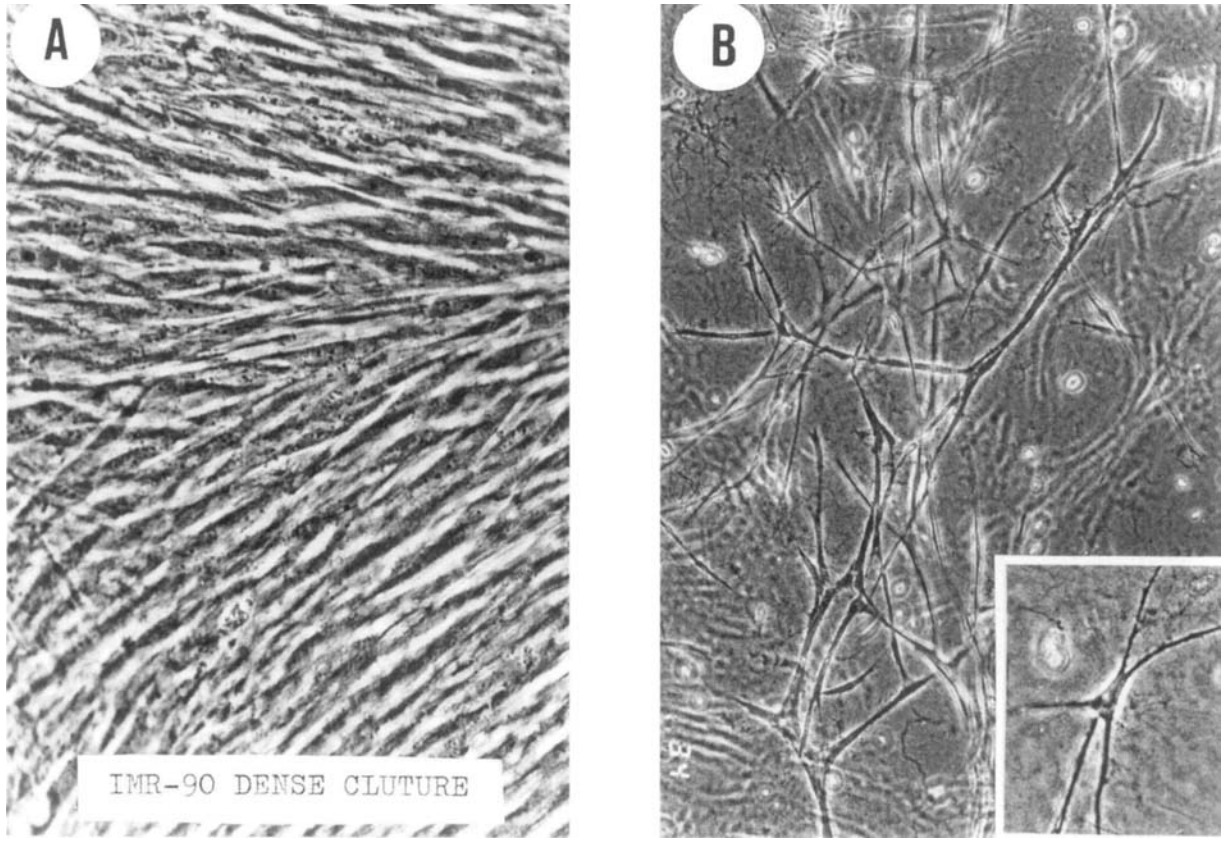

Fig. 1. A) Phase contrast microphotograph of a dense contact inhibited human diploid fibroblast strain IMR-90 grown conventionally as a monolayer in plastic culture dishes. B) The same cells grown in collagen gel prepared with the same medium. Inset shows a single cell at higher magnification. Both cultures were plated at identical density. The population doubling time in collagen gel was significantly greater.

trypsin-EDTA were counted microscopically using Neubauer cell chambers and then plated in 60 mm Petri dishes (Falcon) either in collagen gel semi-solid medium or in liquid medium. The number of cells plated was usually adjusted so as to obtain around 100 colonies per dish after compensating for radiation killing. The maximum number of cells inoculated per dish after compensating for radiation killing never exceeded 4×10^4, in order to minimize feeder effects. Colonies growing on plastic were fixed and stained with crystal violet before counting. For V79 cells, optimal colony-scoring was done on the fifth or sixth day by projection of the colony image onto a sheet of white paper. IMR-90 colonies were assayed after 3 weeks of incubation by counting colonies of 50 or more cells under a dissection microscope. Colony counts in gel cultures were obtained after fixing the colonies by pipetting a layer (2–3 ml) of 4% glutaraldehyde in PBS (phosphate-buffered saline) onto the gel. Counting was done under a dissection microscope. V79 colonies in gel were scored at about the seventh day. IMR-90 required 6 weeks' incubation before scoring could be done. A longer incubation period is required for diploid cell cultures in collagen gel because of the slower growth rate.

RESULTS

Anchored cells: collagen gel vs. plastic

Provision of a natural ECM such as fibrillar collagen changes the phenotype of euploid cells like IMR-90 profoundly (see Fig. 1). Fig. 1A shows the morphology of IMR-90 monolayers which is generally known, when the culture becomes dense and contact-inhibited. The cells in such dense cultures are spindle shaped and develop whorls of overlapping cells. The picture shows an area between the whorls where the cells may criss-cross. Fig. 1B shows correspondingly dense contact-inhibited IMR-90 cells in collagen gel culture. The phenotype of the latter is completely different, characterized by the profuse development of filopodia as exemplified by the inset. The cells in such cultures grow in a three-dimensional matrix by attaching to the fibrillar mesh provided by polymerized collagen.

Fig. 2 presents the corresponding radiation responses of IMR-90 cells plated either directly onto plastic or in collagen gel. The cultures were irradiated after attachment. The survival curves clearly indicate a change in

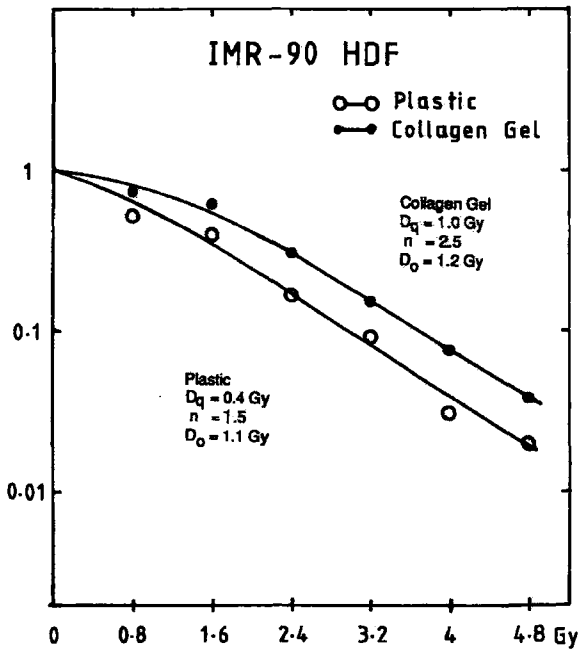

Fig. 2. Clonogenic survival of IMR-90 cells plated either in collagen gel or on plastic before irradiation. Improved survival in gel is due to the broader shoulder. Estimates of D_0 and n by multivariate least-squares regression analysis gave $D_0 = 1.20 \pm 0.01$, $n = 2.49$ (2.4–2.59) for collagen gel and $D_0 = 1.12 \pm 0.06$, $n = 1.45$ (1.31–1.94) for plastic.

response marked by improved survival of clonogens growing in collagen. The improved survival is attributable to the higher D_q value [$D_q = D_0 \cdot \ln(n)$], which is essentially the manifestation of a distinct shoulder on the survival curve. The same experiment done with V79, which is a transformed cell line, yielded opposite results. The survival curves in Fig. 3 show reduced clonogenicity or a sensitization of the cells growing in collagen, which may be ascribed to the reduced shoulder and corresponding D_q value. This contradictory finding is, however, explainable. The V79 cells which were plated in collagen gel did not attach to the fibrils. Being unable to attach, the cells remained rounded (as after trypsinization) and were therefore irradiated in this state. These V79 cells have apparently lost their natural affinity towards collagen. As can be seen in Fig. 4B, surviving V79 clonogens in collagen gel developed compact colonies which were in fact spheroids. Collagen gel in this case behaved like agar used in semi-solid media for clonogenic assays in suspension. The outgrowth of cells from one colony which was near to the bottom plastic surface can be recognized. Fig. 4A shows a culture of similar age grown on plastic. Individual cells cannot be recognized because of piling and the accumulation of cell debris.

Disruption of cell anchorage

Fig. 5 demonstrates the effect of trypsin treatment on the survival of V79 clonogens after irradiation. Trypsin treatment caused cellular sensitization ascribed to the lower D_q value. The analogy between this treatment and that of V79 cells in collagen gel will be discussed. Although a short exposure to trypsin caused cytoplasmic retraction which appeared similar to that of cytochalasin B treatment, the trypsin effect could not be as readily reversed. These cells required 2–3 h to respread compared with 10–15 min after cytochalasin treatment. The resemblance in microscopic appearance is, therefore, deceptive. Obviously, different mechanisms of action are involved. The opposite effects of trypsin and cytochalasin (vide infra) on clonogenic survival provide evidence in support of this.

Cytoskeletal poisons: cytochalasin B vs. phalloidin

Cytochalasin B is a specific cytoskeletal poison which prevents the polymerization of actin into microfilaments. Exposure to cytochalasin B caused a rapid retraction of the cytoplasm, which was as rapidly reversed upon removal of the substance. Fig. 6A shows V79 cells at early confluence, while Fig. 6B shows such cells after 5–10 min

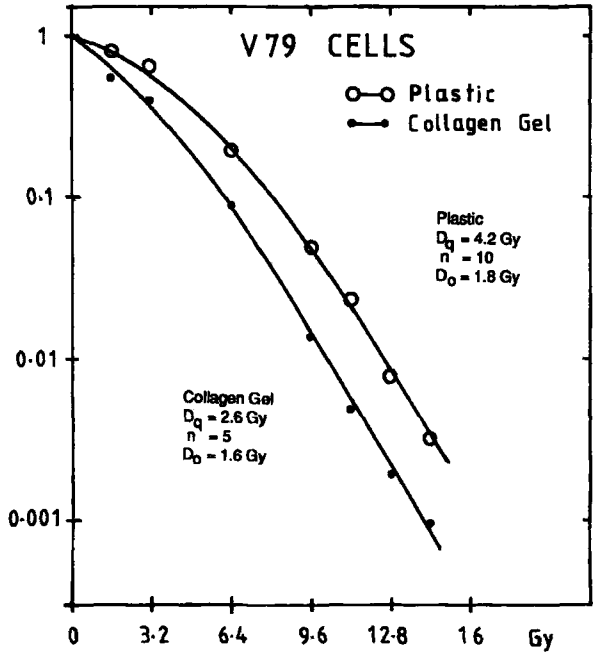

Fig. 3. Clonogenic survival of V79 cells plated either in collagen gel or on plastic before irradiation. Reduced survival in gel is due to the narrower shoulder. Estimates of D_0 and n by multivariate least-squares regression analysis gave $D_0 = 1.63 \pm 0.12$, $n = 5$ (2.91–11.30) for collagen gel and $D_0 = 1.84 \pm 0.11$, $n = 10$ (7.17–15.56) for plastic.

exposure to cytochalasin B at 10 mg/ml. Irradiation in the retracted state immediately after removal of cytochalasin B gave increased survival of clonogens as shown in Fig. 7. The conferred radioresistance is obviously due to the increased D_0 value. The radioprotective effect on the linear exponential part of the survival curve is dramatic. There was no effect on D_q.

The stabilization of microfilaments by blocking their depolymerization into actin through analogous treatment with phalloidin did not elicit any discernible morphological changes. Phalloidin treatment did not provoke any change in radiation response. The survival data were not significantly different from those of the running control and are presented in Table 1.

Cytoskeletal poisons: nocodazole vs. taxol

The other prominent constituent of the cytoskeleton is the microtubular system which can also be either stabilized or destabilized by treatment with the corresponding cytoskeletal poison. Nocodazole is analogous to cytochalasin B in that it prevents the polymerization of tubulin into microtubules, while taxol is analogous to phalloidin in that it prevents the depolymerization of microtubules into tubulin. Treatment of cells with either nocodazole or taxol at the concentration mentioned did not cause cytopathological changes in interphase cells, although it was sufficient to induce mitotic block in either case. Under these conditions, neither of the treatments affected clonogenic survival. The survival curves were identical to the corresponding running controls. The survival data are presented in the table.

DISCUSSION

Cell phenotype

In developmental biology it is well known that the expression of cell phenotypes is not just dependent on gene programmes but also on cellular environmental factors (epigenetics). Although the cell types used in this study differ in origin (human and hamster), histologically they are both fibrocytes. Furthermore, IMR-90 is a non-immortalized cell strain, whereas V79 is an immortalized and

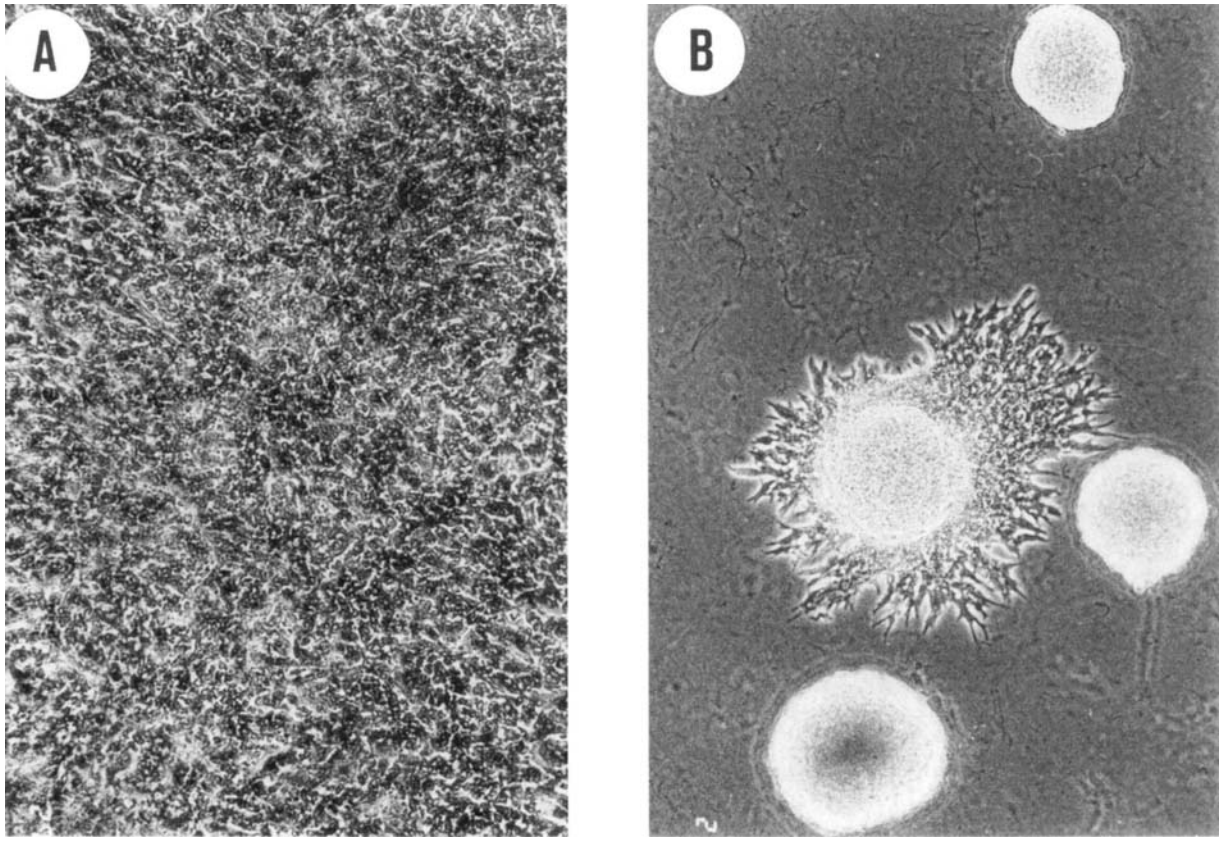

Fig. 4. A) Phase contrast microphotograph of a dense V79 culture grown (one week) on plastic. In contrast to IMR-90, individual cells are not recognizable because of piling. B) A parallel culture in collagen gel produced spheroidal colonies as if the cells were in agar. The clonogens obviously failed to attach to the collagen fibrils, indicating their inability to interact with collagen. Cells from such a colony near the plastic surface are seen to have grown out and spread onto the plastic surface.

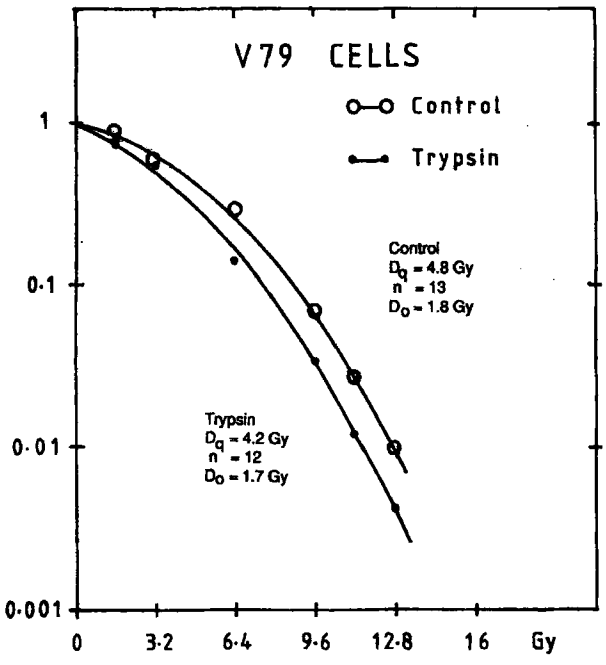

Fig. 5. Clonogenic survival of V79 cells treated with trypsin to cause partial detachment by ECM destruction before irradiation. Reduced survival is due to the narrower shoulder. Estimates of D_0 and n by multivariate least-squares regression analysis gave $D_0 = 1.82 \pm 0.15$, $n = 13$ (3.5–31) for the running control and $D_0 = 1.74 \pm 0.15$, $n = 12$ (3.8–33) for trypsin treatment.

transformed cell line. With the exception of simple epithelia, all other cell types in vivo grow in three-dimensional matrices composed of polymerized macromolecules, collagen being the universal primary component. In contrast to IMR-90, the inertness of V79 cells to collagen indicate loss of natural affinity towards collagen fibrils.

Cell phenotype and radiation response

A correlation between induced cell phenotype and radiosensitivity has been established in this study. It has also been shown that phenotypic expression depends on specific features of the ECM and cytoskeleton. IMR-90 cells acquired improved clonogenic survival correlated with a multifilopodial phenotype when grown in collagen gel. This phenotype is certainly a better reflection of the morphology of fibroblasts in vivo. The radioresistance acquired is due to the higher D_q value which increased the shoulder of the survival curve. Other studies have shown that the survival curves of human diploid fibroblasts in vitro typically lack shoulders (14, 15). These data also suggest that dicrepancies concerning radiosensitivities of human fibroblasts based on in vivo observations (radiotherapeutic experience) and from in vitro experimental results may to a yet undefined extent be due to the inadequacy of commonly used two-dimensional cell-culture systems, which fail to provide adequate microenvi-

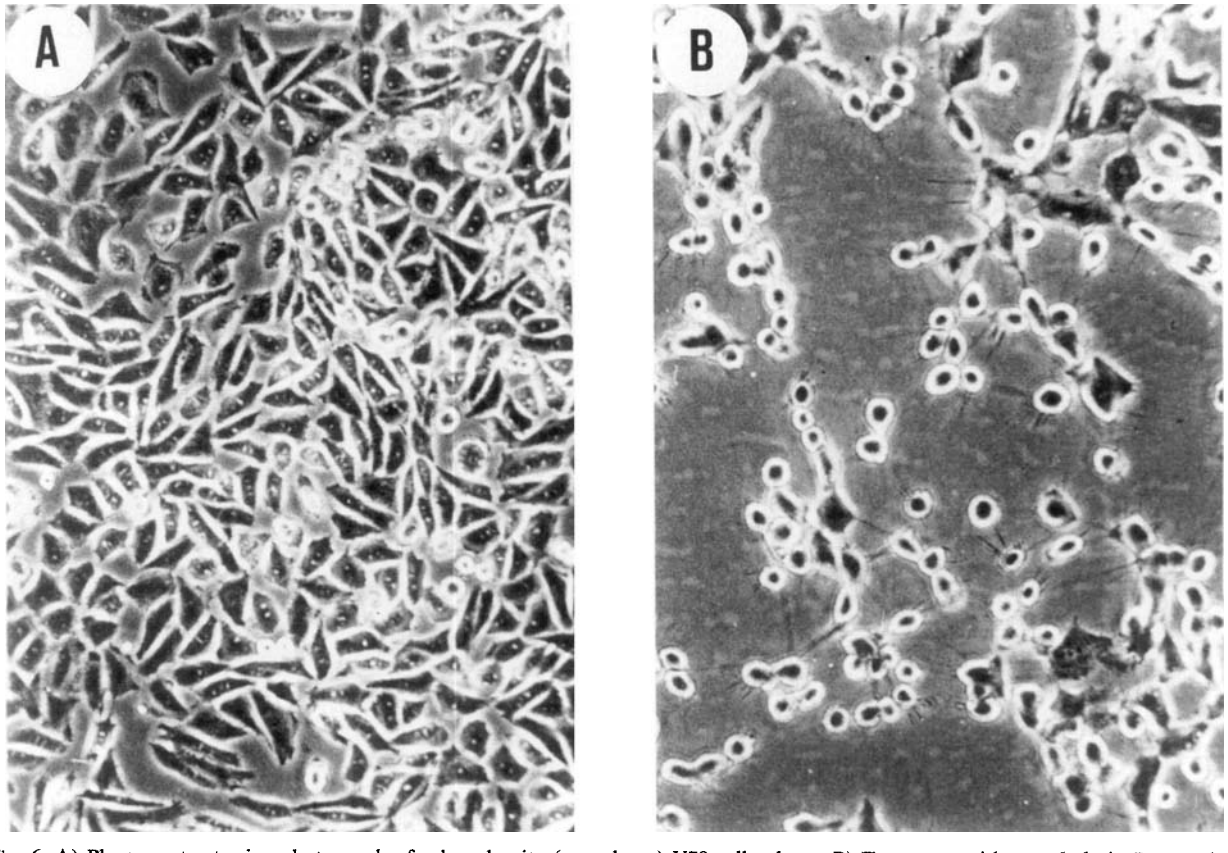

Fig. 6. A) Phase contrast microphotograph of a low density (monolayer) V79 cell culture. B) Treatment with cytochalasin B provoked cytoplasmic retraction which is reversible. Both retraction and reversal can be induced within 10–15 min.

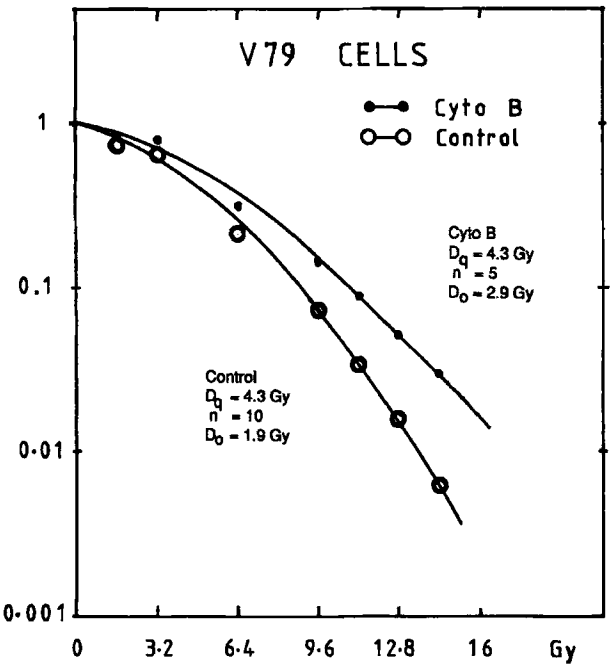

Fig. 7. Clonogenic survival of V79 cells after treatment with cytochalasin B followed by irradiation while the cells were still in the rounded state. Improved survival is seen on the linear exponential part. Estimates of D_0 and n by multivariate least-squares linear regression gave $D_0 = 2.91 \pm 0.34$, $n = 5.2$ (1.63–16.52) for cytochalasin B treatment and $D_0 = 1.94 \pm 0.19$, $n = 10.4$ (3.5–30.0) for the running control.

ronmental conditions, especially three-dimensional anchorage.

On the other hand, transformed cells, such as the V79 cells used in this study, have obviously been so thoroughly adapted to growth on the surface of culture dishes, that the natural affinity towards collagen has been lost. After trypsinization and plating into collagen gel, these cells failed to attach to the fibrillar network. They therefore remained rounded and in a radio-sensitized state, as indicated by the large reduction in D_q. The size of the shoulder is generally regarded as an index of the recovery capacity of cells.

Destruction of the ECM

Being unspecific in its action, trypsin is an enzyme which releases cells from their environment by destroying the ECM. Since collagen as well as other components of the ECM get broken down, cells lose anchorage. This results in the well-known rounding of cells. Electron microscopic examination reveals the withdrawal and falling together of cytoplasmic processes on the cell surface, while within the cell disorder of cytoskeletal elements can be seen, indicating cytoskeletal collapse (16). As redeveloping attachment sites must involve major cellular events accompanied by reorganization of the cytoskeleton, the spreading of cells after trypsin application takes hours before a complete recovery is attained. In this situation cells are in a radiosensitized state obviously because of impaired recovery/repair capabilities, as indicated by the decrease in D_q value in the experiment on timed trypsin exposure without cell detachment. The experiment in which V79 cells were highly radiosensitized when plated into collagen gel can also be viewed as one of protracted trypsin effect—cells have to be trypsinized before they can be plated—since these cells could anchor and spread. The extent of the effect in the timed exposure without detachment was less (lower D_q reduction) probably because trypsin action was interrupted to prevent total detachment.

Manipulation of cytoskeletal elements

Two out of six cytoskeletal poisons tested for cytotoxicity, cytochalasin D and colchicine, were excluded because of poor reversibility of effects. The four selected poisons are in fact two pairs of antagonistic agents. The essential mode

Table 1

The parameters defining radiosensitivity D_0, n and D_q obtained from the survival curves of V79 cells after treatment with phalloidin, nocodazole and taxol, respectively, are summarized here. As DMSO was generally used for preparing stock solutions to dissolve the cytoskeletal poisons, its eventual influence at the final concentration was also checked. The survival curves for all four different treatments were identical to their respective running controls

Parameter	Substance	DMSO	Phalloidin	Nocodazole	Taxol
Running Controls	D_0	1.64	1.83	1.80	1.60
	n	10	10	13	12
	D_q	3.72	4.21	4.82	3.72
Treated	D_0	1.64	1.82	1.80	1.60
	n	10	9	13	12
	D_q	3.72	4.02	4.82	3.72

of action is either the prevention of molecular polymerization, which leads to formation of the respective cytoskeletal elements, or, in contrast, the prevention of break-down or depolymerization of the respective cytoskeletal elements. Cytochalasin B and phalloidin specifically affect the microfilaments, whereas nocodazole and taxol specifically affect the microtubuli.

With the exception of cytochalasin B, the remaining three substances had no effects on the clonogenic survival of the irradiated V79 cells. Treatment with cytochalasin B improved clonogenic survival significantly, but in a peculiar way: the effect was restricted to the linear-exponential part of the survival curve, i.e. D_0 was raised without affecting D_q. As was seen in those cases where survival was affected, D_q was correspondingly raised or lowered, as the case might be. As mentioned above, the value of D_q has generally been regarded as a measure of cellular ability to recover from/repair radiation damage. Concomitantly, shifts in D_0 are now generally regarded as an indication of change in inherent cellular radiosensitivity. In keeping with this, one can envisage conformational changes linked with the packing of nuclear chromatic material. The question is, of course, how a specific microfilament poison could influence the status of chromatic material in the nucleus. Nothwithstanding others, there are two mechanisms by which this was made possible: (a) that cytochalasin B directly affects the nuclear matrix/scaffold which to a major extent is comprised of actin, and (b) that the cytoplasmic filaments are linked to the nuclear scaffold and that destabilization of these filaments in turn affects the nuclear scaffold. The acquired radioresistance is probably not due to improved repair rates but could instead be the result of conformational changes in chromatin which favour the elimination of lethal lesions, e.g. double strand breaks (DSBs) via changed durations of availability of DSBs for repair.

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