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Original Paper

Reversion of UVC-induced Tumorigenic Human Hybrid Cells to the Non-tumorigenic Phenotype

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Non-tumorigenic HeLa × skin fibroblast human hybrid cells were UVC-irradiated (10 J/m^2) and induced to neoplastic transformation with accompanying morphological change and expression of the HeLa tumour-associated antigen, intestinal alkaline phosphatase (IAP). A single-cell-derived cell line was cloned out of a neoplastically transformed focus and designated as UV-12. In low density culture, this cell line demonstrated the ability to undergo reversion to a morphology similar to that of the non-tumorigenic parent with accompanying, much reduced levels of IAP expression. The frequency of this reversion to low IAP expression increased with passage of low density cultures reaching 10^{-2} at 26 passages. A revertant colony was selected and expanded into a cell line which was designated UV-12-RM-1. This cell line had a 67-fold reduction in IAP expression compared to UV-12 and demonstrated a much reduced tumorigenic phenotype. A cell line reconstituted from a tumour derived from this cell line demonstrated a high IAP expression level (3-fold less than UV-12) and was highly tumorigenic. Six single-cell-derived lines were cloned from UV-12-RM-1 and all had low IAP expression. Of these, one demonstrated an aggressive tumorigenicity, four showed the reduced tumorigenic phenotype characteristic of UV-12-RM-1, and one (UV-12-RM-105) was non-tumorigenic. However, with passage in culture, this latter cell line reverted to a weakly tumorigenic phenotype and a much elevated IAP level. It is hypothesised that the phenotypic shifts demonstrated by these UV-induced tumorigenic cells are under epigenetic control, and that they are most likely a consequence of an underlying genetic instability in the survivors of UVC-irradiation.

Key words: morphologic and tumorigenic reversion, genetic instability, epigenetics
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INTRODUCTION

THE MOLECULAR genetics of cancer progression is a rapidly evolving subject [1–3]. Central to this topic is the theme of multiple genetic alterations involving both the activation of cellular proto-oncogenes (e.g. by point mutation or chromosomal translocation) and the inactivation of tumour suppressor genes (e.g. by point mutation, deletion or whole chromosome loss). The role of epigenetic phenomena in cancer development is somewhat more controversial, yet there does exist strong evidence for it [4]. Whereas the genetic basis presupposes a mutational event, epigenetics assumes a loss of control involved in normal differentiation and how this is affected by physiological constraints of growth on metabolism. Intimately involved in both genetic and epigenetic processes is the phenomenon of genomic instability. Indeed, it has been argued that an early step in tumour progression is the induction of a mutator phenotype which results in genetic instability [5]. This, in turn, can result in adaptive responses which are also part of the carcinogenic process [6].

There is considerable evidence for a role in epigenetic phenomena in radiation-induced neoplastic transformation of cells *in vitro* [4]. The influence of modification of the post-irradiation environmental factors (cell density, tumour promoters, protease inhibitors) on the frequency of neoplastic transformation is well established [7, 8]. The delayed expression of heritable, potentially transforming damage is also thought to be influenced by physiological conditions [9]. In addition, there is evidence that radiation-induced and spontaneously arising neoplastically transformed cells can revert to a non-tumorigenic phenotype when grown at low density in optimal growth media [10–12].

In this paper, we describe an interesting series of observations on the reversion of a UVC-induced tumorigenic Hela

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× skin fibroblast cell line to the non-tumorigenic phenotype. This cell system is uniquely suited to *in vitro* studies of neoplastic transformation since it has a unique marker for neoplastic transformation in the expression of the HeLa tumour-associated antigen, intestinal alkaline phosphatase, IAP [13], and a specific molecular target in the putative HeLa tumour suppressor gene located on chromosome 11 [14]. It is currently the only human-derived cell system available for detailed quantitative studies of transformation from the non-tumorigenic to the tumorigenic phenotype [15]. Experimental results which support a role for both genetic and epigenetic processes in the neoplastic transformation of these cells are highlighted and, finally, a dynamic model for late events involved in this process is developed.

MATERIALS AND METHODS

Cell lines

The cell lines used in this study were the non-tumorigenic HeLa × skin fibroblast human hybrid cell line designated as CGL1 [16] and several lines cloned from a UVC-induced tumorigenic segregant of this cell line. The derivation of these cell lines is outlined in Figure 1. CGL1 was derived from the third serial subclone in methylcellulose of the hybrid line ESH5 and is particularly stable against spontaneous neoplastic transformation (transformation frequency $<10^{-5}$). Cells were grown in Auto-Pow minimal essential medium supplemented with 5% calf serum, 2 mM L-glutamine, non-essential amino acids, 100 IU/ml penicillin and buffered with 20 mM sodium bicarbonate. Cultures were routinely tested for the presence of mycoplasma [17].

UVC-irradiation

UVC-irradiation was provided by a short wave, 4 W ultraviolet lamp (model EF140-C, Spectronics Corporation, Westbury, New York, U.S.A.). The fluence of 254 nm light used was 10 J/m²/min. Previous experiments have demonstrated that this fluence induces neoplastic transformants with a frequency of 2×10^{-4}–3×10^{-4} [18]. Dosimetry was carried out using a digital radiometer with a 250 nm sensor (model DM-254-N, Spectronics Corporation, Westbury, New York, U.S.A.). Confluent cultures of cells were exposed in 100 mm Petri dishes following removal of the coverslip and overlying medium. Following irradiation, fresh medium was returned to the cells and they were placed in a humidifed air–CO_2 incubator at 37°C for 6 h. The cells were then put into suspension and plated into T-75 flasks at a density of approximately 3×10^3 cells/flask and returned to the incubator.

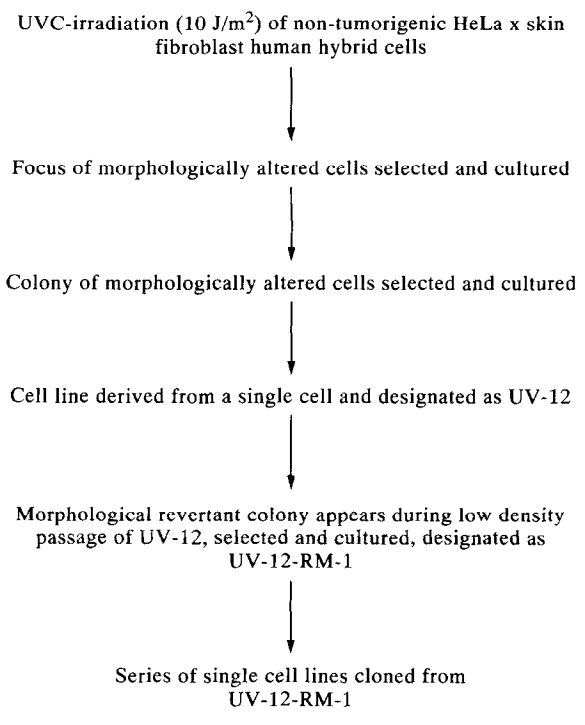


Figure 1. Schematic of the development of the UV-12 cell lines.

Isolation of IAP positive cells

The cultures of UVC-irradiated cells were screened on daily basis for the appearance of colonies with a morphology characteristic of tumorigenic segregants of these cells [9, 19]. Several such colonies were identified, selected and sub-cultured, and the resulting cultures tested for expression of the HeLa tumour-associated antigen, p75/150 [20], using the BD-6 monoclonal antibody and immunoperoxidase staining [15, 21]. This antigen has subsequently been identified as intestinal alkaline phosphatase, IAP [13]. Cultures testing positive were then further subcultured until homogeneously staining populations were obtained. From such cell populations, single-cell-derived cell lines were then cloned. One such cell line was designated as UV-12 and was utilised in the current study.

Assay of AP activity

Alkaline phosphatase (AP) activity of attached monolayers was quantitated with a standard kit from Sigma Chemical Company (Sigma No. 245) by determining the rate of hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol which can be monitored spectrophotometrically at 405 nm. Briefly, 0.6 ml of *p*-nitrophenyl phosphate (16 mM) was added to a monolayer of cells in a well of a 24-well plate. At various times thereafter the supernatant was sampled, appropriately diluted and the absorbance at 405 nm measured. The results are expressed as change in absorbance per minute and are normalised to 10^5 cells and a dilution of 1:50. The activity measured actually represents IAP since it has previously been shown that this is the only form of AP expressed in these cells [13, 22].

Tumorigenicity testing

Cell lines of interest were expanded, and single cell suspensions were prepared so that the final concentration of cells was 1×10^7 cells/0.2 ml of growth medium. Tumorigenicity of the cell lines was tested by injecting 10^7 cells per site subcutaneously into athymic *nu/nu* mice on the right and left flanks. Six sites (two per mouse) were injected per cell line. The injection sites were examined twice weekly and the length, width and height of any emerging tumours was measured and tumour volumes calculated. The average volume of all tumour sites was plotted against time after injection, and the tumour volume doubling time and the time to reach a volume of 500 mm³ were determined.

Tumour reconstitution

Tumours were reconstituted into cell lines by standard procedures, details of which have previously been published [22].

Selection of reverse mutants

In the course of routine passage of the cell line UV-12, colonies exhibiting a morphological reversion to the non-tumorigenic phenotype were apparent at a low frequency. One such colony was isolated and a cell line derived which was designated as UV-12-RM-1. Subsequently, a series of single-cell-derived cell lines were isolated from this colony-derived cell line and designated as UV-12-RM-101, -102, -103, etc.

Determination of frequency of reversion to low AP expression

A single-cell subclone of UV-12 was isolated and a population grown up and plated at low cell density (200 cells/flask) into 10 T-25 flasks and at higher cell density (10^5 cells per T-25 flask). This latter flask was used for routine passaging of the cells (twice per week). At no time did these cultures ever get overcrowded; a confluent culture of UV-12 in a T-25 flask contains 4×10^6–5×10^6 cells. At various time intervals (i.e. passages), the cells were again plated out into 10 T-25 flasks at low cell density for colony formation. After 7–9 days incubation, these flasks were stained with the alkaline phosphatase specific stain Western blue (WB) and the stained colonies counted. The flasks were then restained with crystal violet and again colonies were counted. The reversion frequency was calculated as the fraction of colonies which were WB, that is AP, negative.

RESULTS

The paragraphs below describe the characterisation at the cellular level of a series of cell lines derived from UVC-irradiated non-tumorigenic HeLa × skin fibroblast human hybrid cells. The derivation of these cell lines is outlined in Figure 1. In the context of the results to be described, it is important to note that UV-12 is a single-cell-derived tumorigenic cell line; UV-12-RM-1 is a colony-derived morphological revertant with features of the parent non-tumorigenic cell line, CGL1; and the UV-12-RM-100 series are single-cell-derived subclones of UV-12-RM-1.

UV-12 characterisation

Colonies of UV-12 cells showed the compact morphology typical of many tumorigenic segregants of these human hybrid cells and is distinctly different from that of the parental cell line, CGL1 (Figure 2). Furthermore, these cells stained strongly positive for expression of the HeLa tumour-associated antigen p75/150 (Figure 2), possessed a high AP activity (Table 1) and were highly tumorigenic, growing tumours rapidly at all injected sites (Table 1). Furthermore, restriction fragment length polymorphism (RFLP) analysis, using the highly informative probes HRAS1 (11p15.5) and APOA1 (11q23–24), indicated no evidence for loss of either copy of fibroblast chromosome 11 (data not shown). This is in contrast to gamma-induced tumorigenic cells from this same parent where five of eight cell lines showed evidence of loss of a complete copy of fibroblast chromosome 11 [23]. This observation is consistent with the propensity of ionising radiation to induce major deletions whereas UVC principally induces point mutations.

UV-12-RM-1 characterisation

UV-12-RM-1 was derived from a colony which exhibited a morphological reversion (Figure 2) and negative staining for p75/150 and a low AP activity (Table 1). This cell line also had reduced tumorigenicity as determined by both the number of sites which grew tumours and the time to reach 500 mm^3 in volume. The frequency of reversion of UV-12 to low AP expression was determined as a function of time in culture (Figure 3). It can be seen that the reversion frequency increased with time in culture, approaching 10^{-2} at 90 days (approximately 26 passages). This reversion was always accompanied by a change in morphology from the very compact colonies of cuboidal cells typical of UV-12 to more diffuse colonies consisting of larger, more elongated cells.

UV-12-RM-1 tumour reconstitutes

The tumour reconstitutes of UV-12-RM-1 demonstrated a phenotype similar to that of UV-12 in terms of morphology, tumorigenicity and AP expression (Table 1). This result would be consistent with the hypothesis that UV-12-RM-1, a colony-derived cell line, was contaminated with a small subpopulation of UV-12 cells which were responsible for the tumour formation from UV-12-RM-1. However, an alternate explanation could be that further cellular alterations were occurring *in vivo* to promote the reversion of the cells to a more tumorigenic phenotype, or both of these possibilities could be operational. Consequently, a series of single-cell-derived cell lines were isolated from UV-12-RM-1. If the former hypothesis were true, then some of these cell lines could be non-tumorigenic.

UV-12-RM-1 subclones

Six single-cell-derived cell lines were isolated from UV-12-RM-1 and tested for AP activity and tumorigenicity (Table 1). One of these cell lines, RM-106, demonstrated an aggressive tumorigenic phenotype characteristic of UV-12. Four of the lines, RMs 101, 102, 103 and 104, demonstrated the much less aggressive tumorigenic phenotype typical of UV-12-RM-1, with less than 100% of injected sites growing tumours, and the tumours that did grow often showed variable growth kinetics (Figure 4). All of these lines had AP activities of <3% of UV-12. An early passage of UV-12-RM-105 exhibited an AP activity of 0.7% of UV-12 yet did not grow tumours in any of six sites followed for 250 days. However, a later passage of this cell line demonstrated an intermediate tumorigenicity, growing tumours in three of six sites, while possessing a much increased AP activity (45% of UV-12).

UV-12-RM-100 series tumour reconstitutes

Two examples of the tumorigenicity of the tumour reconstitutes of the RM-100 series, RM-101-0R and RM-102-2R, are given in Table 1. It can be seen that, unlike their parent cell lines, both of these reconstitutes demonstrate rapid tumour formation at all injected sites. Furthermore, while RM-101-0R also shows selection for a much enhanced AP activity, this is not the case for RM-102-2R. Thus, this selection for the rapid growth phenotype is not connected with the level of AP activity, an observation which is consistent with previous findings [22].

DISCUSSION

The results presented in this paper demonstrate that UV-induced tumorigenic HeLa × skin fibroblast human hybrid cells can revert *in vitro* to a less aggressive tumorigenic phenotype and even to the non-tumorigenic state. Such reversion has previously been observed to occur spontaneously under appropriate physiological conditions in several tumorigenic mouse cell lines [11, 12] and in X-ray-induced neoplastically transformed C3H10T1/2 cells [10]. The frequency of this

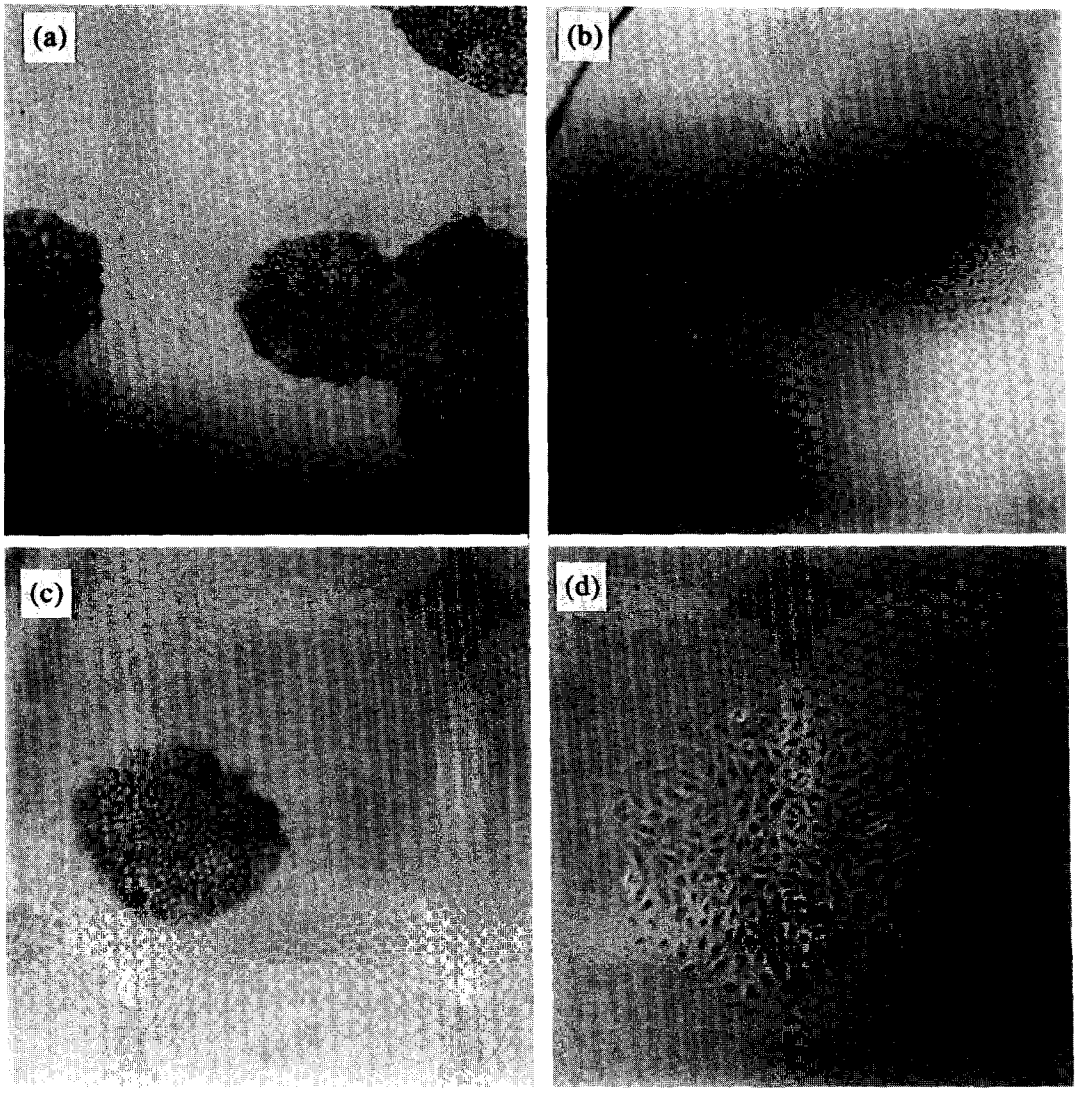


Figure 2. Photomicrographs illustrating changes in colony morphology and immunoperoxidase staining for the HeLa tumour-associated antigen. (a) UV-12, note the uniform morphology and intense staining. (b) A colony of UV-12 with an obvious revertant colony exhibiting a much reduced staining intensity and reversion to a morphology characteristic of the non-tumorigenic parent cell line, CGL1. (c) and (d) Close-up views of the colonies shown in (b).

reversion increases with time in culture when the cells are kept under optimal growth conditions over a long period of time. However, the revertants characterised here are of themselves not stable and can back-revert *in vitro* to a more tumorigenic phenotype. Selection for a more aggressive tumorigenic phenotype also occurs *in vivo*, again in agreement with previous findings [22]. This could simply be either the selection of a subpopulation of the injected cells or the *in vivo* conversion of the general population.

In order to attempt to explain the mechanism underlying these observations, it is relevant to discuss the current hypothesis as to the mechanism underlying the neoplastic transformation of these cells and its relationship to the expression of IAP. The use of somatic cell hybrids in the study of tumour suppression is well established. One of the most extensively studied human hybrid cell systems is that of HeLa × skin fibroblasts [19]. These cells are transformed (i.e. are immortal and lack contact inhibition of growth) but not tumorigenic. Their chromosomes are typically stable (modal chromosome number of 96–100) and karyotypic analysis of rare spontaneous tumorigenic segregants reveals a loss of one copy of chromosomes 11 and 14 [16]. The tumorigenic segregants also express the HeLa tumour-associated antigen, p75/150—intestinal alkaline phosphatase [13, 20]. Microcell-mediated transfer of a single copy of human chromosome 11 into tumorigenic segregants results in a reversion to the non-tumorigenic phenotype and suppression of expression of p75/150—intestinal alkaline phosphatase [14]. However, transfection of the *IAP* gene into non-tumorigenic hybrids, with resulting high expression of IAP, did not confer tumorigenicity [24]. Nor did a similar transfection into a weakly tumorigenic, low IAP expressing, gamma-induced tumorigenic cell line result in any increase in tumorigenicity [22]. Based on these observations, it has been concluded that IAP expression is necessary but not sufficient to confer the tumorigenic phenotype and that a common regulator of *IAP* and an as yet unidentified oncogene resides on chromosome 11 [22–24].

Table 1. *Tumorigenicity, tumour growth kinetics and alkaline phosphatase activity in UVC-induced tumorigenic HeLa × skin fibroblast human hybrid cells*

Cell line	Tumorigenicity	Range of time to 500 mm³ (days)*	IAP activity (relative units)
CGL1	0/100	—	0.0012
UV-12	6/6	21–31	1.0†
UV-12-RM-1	4/6	63–73	0.015
UV-12-RM-1-R	6/6	11–15	0.33
UV-12-RM-101	2/6	80–90	0.001
UV-12-RM-102	4/6	40–78	0.0017
UV-12-RM-103	3/6	52–58	0.0067
UV-12-RM-104	3/6	44–69	0.0034
UV-12-RM-105E	0/6	—	0.0072
UV-12-RM-105L	3/6	30–40	0.45
UV-12-RM-106	6/6	13–24	0.023
UV-12-RM-101-OR	4/4	15	0.38
UV-12-RM-102-2R	6/6	9–13	0.010

*Tumour volume doubling time was in the range 5–7 days for all tumours once exponential growth started. †Corresponds to 2.8×10^{-2} absorbance units/min/10^5 cells (see Materials and Methods).

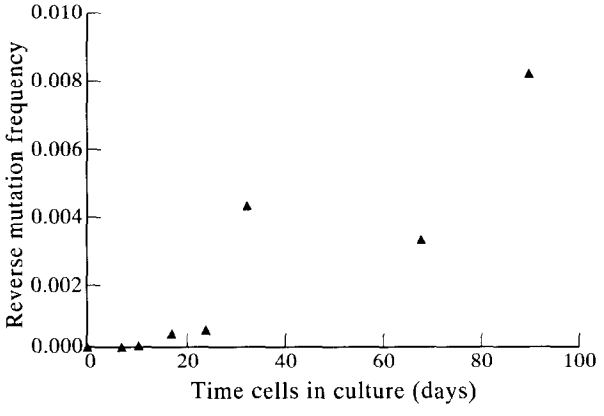


Figure 3. Frequency of reversion to a non-tumorigenic morphology with very low staining intensity for the HeLa tumour-associated antigen as a function of passage in culture.

One mechanism whereby reversion to the non-tumorigenic phenotype could occur could be through the loss of the critical oncogene as a result of genetic instability of these survivors of UVC-irradiation. However, such a mechanism could not account for the reversion to a less tumorigenic phenotype nor the back-reversion to a more tumorigenic phenotype. An alternative explanation therefore has to be sought. It could be that alterations in gene regulation as a consequence of subtle environmentally-induced physiological changes are involved. One possibility could be changes in the methylation status of critical genes. Further investigation is required to test this hypothesis.

It is of interest to examine the relationship between IAP activity in these cells and their tumorigenicity. In general, low activity correlates with reduced tumorigenicity as defined by a longer time to achieve a tumour volume of 500 mm³ and less than 100% of injected sites forming tumours. This is similar to the earlier observation made for gamma-radiation-induced tumorigenic segregants of these hybrid cells [22]. However,

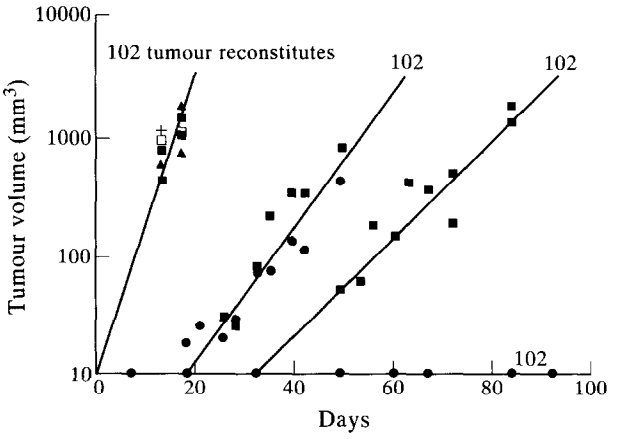


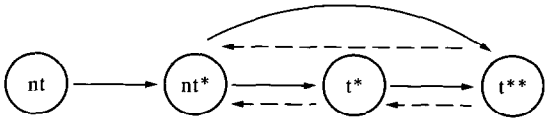
Figure 4. Tumour growth curves for individual sites in *nu/nu* mice injected with UV-12-RM-102 and UV-12-RM-102-2R. Note the heterogeneity of growth kinetics in the initial cell line and the uniformity of growth for the tumour reconstituted cell line.

there are notable exceptions to this pattern. For example, in the present study, UV-12-RM-106 had a relatively low IAP activity yet grew tumours rapidly at all injection sites (Table 1). The same is true for the tumour reconstitute of UV-12-RM-102. In addition, we have made a similar observation with a single-cell-derived tumorigenic cell line isolated from fission-neutron irradiated cells (unpublished data). Similarly, a non-tumorigenic morphology does not necessarily correlate with lack of tumorigenicity [22]. Therefore, from our accumulated experience with these cell lines, one can conclude that, while IAP activity correlates with tumorigenicity, there is no absolute correlation between level of IAP activity and degree of tumorigenicity. Furthermore, our collective experience is that IAP activity is more stable as a function of passage in culture in some cell lines than others, again suggestive of an underlying genetic instability under epigenetic control.

In summary, the data presented demonstrate that UVC-induced tumorigenic HeLa × skin fibroblast human hybrid cells can, with a very low frequency, revert to a phenotype with reduced tumorigenicity and, in one instance, to the non-tumorigenic state. This reversion is characterised by an altered morphology and reduced expression of the HeLa tumour-associated antigen, IAP. Back-reversion of these revertants to a more aggressive tumorigenic phenotype also occurs in these cultures with what would appear to be a much higher frequency. Tumour reconstitutes of weakly tumorigenic cells possess a much stronger tumorigenic phenotype. This is a well recognised phenomenon which is a consequence of selection and adaptation *in vivo*, again providing evidence for the involvement of epigenetic processes [11].

A model is now proposed to account for these observations (Figure 5). This model simply predicts that irradiated cells harbour genetic damage which predisposes instability under certain suboptimal environmental conditions. This genetic instability can result in both the development of the specific damage required for neoplastic transformation, as well as being responsible for the reversion to the non-tumorigenic phenotype or intermediate states. This model would predict that under optimal environmental conditions the neoplastic transformation frequency would be at its lowest. This is borne out not only for irradiated cells but also for non-irradiated

DYNAMIC MODEL OF NEOPLASTIC TRANSFORMATION



nt = non-tumorigenic cell
nt* = nt cell harbouring heritable damage
t* = weakly tumorigenic cell
t** = strongly tumorigenic cell

Movement to the right is favoured under suboptimal conditions of growth (cell crowding, nutrient deprivation, reduced pH) and is more efficient in cells harbouring heritable damage.

Figure 5. Model of cellular pathways which can result in neoplastic transformation and its reversal.

cells in terms of spontaneous neoplastic transformation. The situation for reversion is not as clear, as this may occur under optimal as well as suboptimal growth conditions.

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