SUPPRESSION OF MALIGNANCY IN HYBRID CELLS:
THE MECHANISM

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SUMMARY
When malignant cells, defined by their ability to grow progressively in genetically compatible hosts, are fused with diploid fibroblasts of the same species, the resulting hybrid cells, so long as they retain certain specific chromosomes donated by the diploid parent cell, are non-malignant. When these particular chromosomes are eliminated from the hybrid, the malignant phenotype reappears, and the segregant cell is again able to grow progressively in vivo. In the present experiments the histological character of the lesions produced by the inoculation of crosses between malignant and non-malignant cells was examined. It was found, in a wide range of material, and without exception, that where one or other of the parent cells in the cross was of fibroblastic lineage, malignancy was suppressed when the hybrid cells produced a collagenous extracellular matrix in vivo; and it reappeared when genetic segregants were produced that had lost the ability to produce this matrix. These results are interpreted in terms of a general model in which it is proposed that the progressive multiplication of malignant cells in vivo is a secondary consequence of a genetically stable impairment of terminal differentiation.

INTRODUCTION
It is now well established that when malignant cells, defined by their ability to grow progressively in genetically compatible hosts, are fused with diploid fibroblasts of the same species, the resulting hybrid cells, so long as they retain certain specific chromosomes donated by the diploid parent cell, are non-malignant; and when these particular chromosomes are lost from the hybrid, the malignant phenotype reappears and the segregant cell is again able to grow progressively in vivo (for reviews, see Miller & Miller, 1983; Sager, 1985). This phenomenon, commonly described as the suppression of malignancy or tumorigenicity, has been intensively studied in several laboratories since its discovery in 1969 (Harris et al. 1969), and although substantial clarification of the cytogenetic basis of the effect has been achieved (Jonasson, Povey & Harris, 1977; Evans et al. 1982; Stanbridge, Flandermeyer, Daniels & Nelson-Rees, 1981), little is known about the mechanism underlying it. In this paper, evidence is presented for the conclusion that, in crosses where one or other parent cell is of fibroblastic lineage, malignancy is suppressed when the hybrid cells produce a collagenous extracellular matrix; and it reappears when genetic segregants are produced that have lost the ability to produce this matrix. This finding may have quite general implications for our understanding of how malignant cells are produced.

Key words: malignancy, hybrid cells, extracellular matrix.
MATERIALS AND METHODS

Cells

NIH 3T3 clone D4 and NIH 3T3 clone D4 EJ, a derivative of clone D4 transformed by transfection with a mutated c-H-ras oncogene, were supplied by Dr C. J. Marshall of the Institute of Cancer Research, London. PG19 is a thioguanine-resistant derivative of a mouse melanoma originally arising spontaneously in a C57 Black mouse. In syngeneic, sublethally irradiated newborn recipients, the line produces a 100% take incidence from inocula of $5 \times 10^6$ cells/mouse. Details of the PG19 line and of crosses between PG19 and diploid fibroblasts are given by Jonasson et al. (1977) and Evans et al. (1982). YACIR IMP is a thioguanine-resistant derivative of a lymphoma originally produced in an A.Sn mouse by Moloney virus. YACIR IMP also produces a 100% take incidence from small inocula in syngeneic, sublethally irradiated, new-born recipients. Details of the line, and of hybrids between it and normal fibroblasts are also given by Jonasson et al. (1977) and Evans et al. (1982). A9HT is a highly tumorigenic derivative of the A9 line, produced by passage of the latter through C3H mice. Details of the line, and of crosses between it and diploid fibroblasts are given by Wiener, Klein & Harris (1973). Diploid fibroblasts from C57 Black mice were obtained by trypsinization of 13-day mouse embryos. The cells were used for experiment in passages 3–5.

Cell fusion

Cells were fused by the use of ultraviolet-inactivated Sendai virus, essentially as described by Harris & Watkins (1965). Crosses between PG19 and either untransformed or transformed NIH 3T3 cells were selected in HAT medium, which eliminated the PG19 cells. Hybrid clones could readily be distinguished from NIH 3T3 clones (transformed or untransformed) by their morphology and were isolated by standard techniques. After selection in HAT medium, the cells were grown routinely in Dulbecco's Minimum Essential Medium and 10% foetal calf serum.

Assay for tumorigenicity

The suspension, containing the stated number of cells in a volume of 0.1 ml of phosphate-buffered saline, was injected subcutaneously into the flank of either syngeneic, sublethally irradiated, new-born mice or young adult nude mice, and the animals were examined twice weekly for 3 months.

Histology

Inocula of $5 \times 10^5$ to $10^7$ cells were injected subcutaneously into the flanks of nude mice or syngeneic, sublethally irradiated, new-born mice, and the lesions produced by the injected cells were excised at the stated times up to 15 days after injection. The tissues were fixed in 4% formol saline and processed routinely to paraffin wax. Sections were cut, mounted on glass slides, de-waxed in xylene and taken through an ethanol series to water in the usual way. They were stained with haematoxylin and eosin and with Van Gieson's stain by standard procedures. The preparation of sections, the staining procedure and the conditions of photomicrography were kept constant throughout the investigation. All negatives were printed at the same magnification (given in Fig. 1) and under the same conditions of illumination.

RESULTS

Crosses between a malignant mouse melanoma and NIH 3T3 cells, untransformed and transformed by the H-ras oncogene

The initial purpose of the investigation was to see whether NIH 3T3 cells still retained the ability of their fibroblastic progenitors to suppress malignancy when fused with tumour cells; and if so, whether transformation induced by transfection with the H-ras oncogene had any effect on their ability to do this. Injected
subcutaneously into the flanks of nude mice, untransformed NIH 3T3 cells (clone D4), produce only an occasional tumour even with inocula of $10^7$ cells/mouse. NIH 3T3 cells transformed by transfection with H-ras (clone D4 EJ), give a 100% take incidence with inocula of $10^6$ cells/mouse. The two clones were crossed with the malignant mouse melanoma derivative PG19. From each of the two crosses (PG19 $\times$ transformed NIH 3T3, and PG19 $\times$ untransformed NIH 3T3) four independent clones of hybrid cells were isolated and examined. PG19 gives a 100% take incidence in nude mice with subcutaneous inocula of $10^6$ cells; progressive tumours appear about 7 days after inoculation. Tested in the same way, the PG19 $\times$ transformed NIH 3T3 clones were found to be as malignant as PG19 itself: with subcutaneous inocula of $10^6$ cells/mouse, the four clones produced progressive tumours in 100% of recipients, with mean latent periods of 7, 8, 9 and 9 days. The PG19 $\times$ untransformed NIH 3T3 clones behaved quite differently. The small nodule produced at the injection site by the inoculum itself became progressively smaller and, within two to three weeks, was no longer discernible or palpable. In some animals, however, progressive tumours did appear at the injection site at a much later stage, between 40 and 70 days after the injection of the cells. Extensive experience with these late tumours arising from hybrids in which malignancy was initially suppressed has shown that they are generated by the growth of segregant cells in which the elimination of specific chromosomes derived from the non-malignant parent cell can be demonstrated (Jonasson et al. 1977; Evans et al. 1982). Intraspecific hybrids constructed with NIH 3T3 cells are not favourable material for detailed cytogenetic analysis, but a cursory examination of the tumours produced by the PG19 $\times$ NIH 3T3 and the PG19 $\times$ transformed NIH 3T3 hybrids showed that they confirmed the previous experience. The chromosome constitutions of the tumours produced by the PG19 $\times$ transformed NIH 3T3 hybrids were indistinguishable from those of the hybrid cells inoculated; but in the late tumours arising from the PG19 $\times$ untransformed NIH 3T3 hybrids substantial chromosome losses were observed. It will be demonstrated later that the phenotype of the cells that form these late tumours differs in a decisive way from that of the hybrid cells originally injected.

It is clear from the foregoing experiments that, whereas untransformed NIH 3T3 cells still retain the ability to suppress the tumorigenicity of the PG19 cells with which they are fused, this ability is completely lost when the NIH 3T3 cells are transformed by transfection with the H-ras oncogene. It was therefore of interest to see whether any light on the mechanism underlying this difference between the two sets of hybrid clones might be shed by histological examination of the inoculation site during the period when the malignant (PG19 $\times$ transformed NIH 3T3) hybrids became progressive tumours and the nodules produced by the non-malignant (PG19 $\times$ untransformed NIH 3T3) hybrids regressed. Inocula of $5\times10^6$ to $10^7$ cells of each of the eight hybrid clones were injected subcutaneously into the flanks of nude mice, and the tissues at the sites of injection were removed for examination 5, 10 and 15 days later (12 days in a few cases where the speed of involution of the nodule produced by the PG19 $\times$ untransformed NIH 3T3 hybrids made it unlikely that any recognizable residue of the inoculum would still be present at 15 days). Paraffin
sections were made of the excised tissues and were initially stained with haematoxylin and eosin. In the light of the observations made on these sections, all the material was also stained with Van Gieson's reagent, which marks collagen.

At 5 days the lesions produced by the two sets of clones showed no obvious distinguishing features. In both cases there was usually an area of necrosis at the centre of the inoculum, and this was surrounded by a layer of viable, but overcrowded, cells in which some measure of alignment was nonetheless discernible. Occasional mitotic figures were to be seen in this layer in both sets of clones, and around the periphery of the lesions a small number of inflammatory cells were present. At 10 days, however, the lesions produced by the PG19 X untransformed NIH 3T3 hybrids had undergone a dramatic change. The necrotic centre had usually been resolved, and the surviving cells had begun to produce an extracellular matrix that separated the cell bodies from each other. This extracellular matrix contained recently deposited collagen, which stained a pale pink with Van Gieson's reagent. Very few mitoses were now to be seen. The lesions produced by the PG19 X transformed NIH 3T3 hybrids were now palpable tumours about 0.5 cm in diameter. The cells were pleomorphic, some areas of the tumour having a fibro-blastic character, others a more epithelial character, but there was no evidence of any matrix separating the cells and no extracellular material staining with Van Gieson's reagent. Numerous mitoses were present. At 15 days, the difference between the two sets of clones was even more striking. The non-malignant cells were embedded in a copious collagenous extracellular matrix, which now stained more strongly with Van Gieson's reagent, and mitoses were very rare (Fig. 1). The malignant cells showed numerous mitoses, but still no discernible or stainable extracellular matrix (Fig. 2). This difference between the malignant and the non-malignant hybrids was found without exception in all the clones tested.

While these observations clearly show that the suppression of progressive growth in vivo is associated with the production of a collagenous extracellular matrix, it could be argued that the association is fortuitous. This possibility was effectively eliminated by examination of the histological character of the segregant tumours that arose after a long delay from the inocula of PG19 X untransformed NIH 3T3 hybrids, in which progressive growth was initially suppressed. In the earliest stages examined, the segregant tumour could be seen to arise at the periphery of a remnant of the original inoculum of hybrid cells. These were embedded in an extracellular matrix that stained clearly with Van Gieson's reagent, and there was extensive

Fig. 1. Regressive lesion produced by melanoma PG19 X untransformed NIH 3T3 clone D4 hybrids. The cells are embedded in collagenous extracellular matrix that stains pink with Van Gieson's stain. Bar, 50 µm.

Fig. 2. Progressive tumour produced by PG19 X transformed NIH 3T3 clone D4 EJ hybrids. The tumour shows no stainable extracellular matrix.

Fig. 3. A segregant tumour arising from an inoculum of PG19 X untransformed NIH 3T3 clone D4 hybrids. No extracellular matrix.

Fig. 4. Progressive tumour produced by transformed NIH 3T3 clone D4 EJ. No extracellular matrix.
Suppression of malignancy

fibrosis surrounding the lesion. No mitotic activity was observed in this region. However, at the edge of the original inoculum, a sub-population of cells could readily be identified by its different morphology and by the absence of extracellular matrix. Within this sub-population mitoses were numerous. This growth zone was reminiscent of a mutant papilla on a bacterial colony. At later stages, remnants of the original inoculum were not seen, and the segregant tumours were composed entirely of obviously multiplying cells devoid of any discernible matrix or anything stainable by Van Gieson's reagent (Fig. 3). The histological observations left little doubt that these late tumours were generated by the overgrowth of variants that differed from the bulk of the cells injected in having lost the ability to make a collagenous extracellular matrix.

Like the malignant hybrids to which it gave rise, the transformed NIH 3T3 clone D4 EJ itself produced tumours that were devoid of collagenous extracellular matrix (Fig. 4). Untransformed NIH 3T3 cells were difficult to study in vivo because the great majority of the cells were rapidly killed after injection. Sections at 5 days usually showed nothing but necrotic cells surrounded by an inflammatory reaction. However, in some cases, areas of surviving cells could be found up to the tenth day after injection, and these did show the presence of Van Gieson-stainable extracellular matrix. Large inocula of NIH 3T3 cells injected into nude mice produce occasional progressive tumours. One such tumour arose in the present series of experiments 50 days after injection of the cells. Histological examination revealed that the tumour had a large necrotic centre surrounded by a rim of viable cells showing numerous mitotic figures. These cells, unlike the original NIH 3T3 cells injected, produced no detectable matrix.

In the light of these observations, it is reasonable to propose that untransformed NIH 3T3 cells suppress the tumorigenicity of the melanoma cells because they impose on the hybrids a fibroblastic pattern of terminal differentiation, which involves, among other things, the synthesis of a collagenous extracellular matrix. The NIH 3T3 cells transformed by the H-ras oncogene, on the other hand, having themselves lost the ability to execute this pattern of terminal differentiation, do not confer it on the hybrid cells and thus permit their continued multiplication. On the basis of changes in morphology seen in vivo in hybrids between HeLa cells and diploid human fibroblasts, Stanbridge & Ceredig (1981) have also concluded that

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Fig. 5. Regressive lesion produced by PG19 × A.Sn diploid fibroblast clone 14 hybrids. The lesion shows a necrotic centre surrounded by cells of fibroblastic morphology embedded in collagenous extracellular matrix.

Fig. 6. Regressive lesion produced by diploid fibroblasts (CS7 Black). The cells are separated by collagenous extracellular matrix that stains pink with Van Gieson's stain.

Fig. 7. Regressive lesion produced by lymphoma YACIR IMP" × H-T6T6 diploid fibroblast clone 1G8 hybrids. The cells have fibroblastic morphology and are embedded in collagenous extracellular matrix.

Fig. 8. Progressive tumour produced by malignant subclone 1G8a derived in vitro from the YACIR IMP" × H-T6T6 diploid fibroblast clone 1G8. The cells have a morphology intermediate between that of a sarcoma and that of a lymphoma. There is no extracellular matrix.
suppression of tumorigenicity is associated with terminal differentiation of fibroblastic type. This idea will be discussed in more detail below.

**Crosses between a malignant melanoma and diploid fibroblasts**

How general are these findings? Over the years a large number of crosses between tumour cells and diploid fibroblasts have been constructed in this laboratory; many of them have been subjected to detailed cytogenetic analysis, and their tumorigenicity, or lack of it, extensively tested. A selection was made of the best studied of these hybrids and they were re-examined in the light of the results obtained with the PG19 × NIH 3T3 hybrids. PG19 × A.Sn fibroblast clone 14 (Evans et al. 1982) is, of all the hybrids made here, the one in which malignancy is most stably suppressed. Despite repeated testing over several years it has yet to produce a single tumour. It is exceptional among crosses between tumour cells and diploid fibroblasts in having retained both copies of the diploid chromosome 4, which has been shown to play a decisive role in determining the suppression of the malignant phenotype (Jonasson et al. 1977; Evans et al. 1982). Inocula of 10⁷ cells were injected subcutaneously into the flank of syngeneic, sublethally irradiated new-born C57 Black × A.Sn mice and the histology of the inoculum studied as above. Tests were also done in nude mice. The results were very similar to those obtained with the PG19 × untransformed NIH 3T3 hybrids. The nodules produced by the injection of the hybrid cells regressed rapidly and were no longer palpable in most cases within 15 days. Histological examination of early lesions showed a necrotic centre surrounded by a rim of overcrowded cells. In samples taken at 12 days, however, much of the necrotic material had been resorbed and the hybrid cells, aligned in a typically fibroblastic manner, were now embedded in copious extracellular matrix (Fig. 5). Very few mitoses were seen. Similar results were obtained when the cells were injected into nude mice. In samples removed from these animals at 15 days, the necrotic area had been resorbed, and the residual lesion was composed of fibroblastic cells surrounded by extracellular matrix that stained pink with Van Gieson's reagent, especially at the periphery of the lesion. The fate of diploid fibroblasts (C57 Black), injected as an inoculum of 10⁷ cells into the flank of nude mice, was similar to that of hybrids in which malignancy was suppressed. The nodule produced at the injection site regressed over a period of 10–15 days and was thereafter no longer detectable. Histological examination of the regressing nodule showed the fibroblasts embedded in extracellular matrix that already stained pink with Van Gieson's reagent as early as the fifth day after injection. By the tenth day the fibroblasts were aligned in an orderly fashion and were embedded in collagenous extracellular matrix (Fig. 6). PG19 melanoma cells injected into the flank in the same way produced a rapidly growing, undifferentiated, usually amelanotic tumour free of any stainable extracellular matrix. It is clear that in the case of these PG19 × fibroblast hybrids, failure to grow progressively in vivo is again associated with the production of a collagenous extracellular matrix.
**Suppression of malignancy**

**Crosses between a lymphoma and diploid fibroblasts**

YACIR IMP⁻ × CBA/H-T₅T₆ fibroblast is a cross between a thioguanine-resistant derivative of the Moloney virus-induced lymphoma, YACIR, and diploid fibroblasts from a CBA mouse homozygous for the T₅ translocation. Clones of this cross have been extensively studied (Jonasson et al. 1977; Evans et al. 1982). Clone 1G1 is a highly malignant clone that produced progressive tumours in 90% of syngeneic sublethally irradiated new-born mice with inocula of 5×10⁵ cells/mouse. Clone 1G8 produced no tumours under the same conditions. Clones 1G8a and 1G8b are malignant derivatives of clone 1G8 obtained by selection in agarose and passage through the animal. It was in this material that cytogenetic analysis revealed that the suppression of tumorigenicity in the hybrids involved dosage effects for some gene or genes located on the chromosome 4 derived from the diploid parent cell (Evans et al. 1982). Inocula of 10⁷ cells of clone 1G1, clone 1G8 and of the malignant derivatives, clones 1G8a and 1G8b, were again injected into the flanks of both syngeneic, sublethally irradiated, new-born mice and nude mice, and the histology of the lesions studied. Inocula of clone 1G8 cells produced small nodules at the injection site that regressed slowly over a 15-day period. Nodules removed at 5 days showed a necrotic centre with a rim of fibroblastic cells separated by matrix that stained slightly with Van Gieson’s reagent. By 10 days there was some resorption of the necrotic centre, and the surrounding cells, aligned in a fibroblastic fashion, were now embedded in copious extracellular matrix (Fig. 7). Clone 1G1 and the malignant subclones 1G8a and 1G8b grew rapidly and progressively from the beginning. The lesions were frankly malignant tumours with numerous mitoses, and, in the larger tumours, areas of central necrosis were seen. The morphology of the cells was generally intermediate between that of a sarcoma and that of a lymphoma, although different parts of the one tumour sometimes had a predominantly lymphomatous or predominantly sarcomatous appearance. Segregation of the genetic determinants of fibroblastic and lymphoid morphology in crosses of this type has been previously described (Wiener, Cochran, Klein & Harris, 1972). The tumours produced by clone 1G1 and the malignant subclones of clone 1G8 showed no detectable extracellular matrix (Fig. 8). This is also true of YACIR itself, which produces typical lymphomas whose morphology has been described in detail elsewhere (Wiener et al. 1972). In one animal injected with 10⁷ clone 1G8 cells, after the nodule had remained stationary for 15 days, a progressive tumour appeared. This late tumour, which had a large area of central necrosis, was composed of frankly lymphomatous cells that produced no detectable extracellular matrix. This tumour was obviously a malignant segregant in which segregation of morphological markers had also taken place.

These results make it clear that what has been shown to be true for the melanoma crosses is also true for the lymphoma crosses. When the hybrid cells produce a collagenous extracellular matrix, progressive growth in vivo is suppressed, and when malignant sub-clones are selected from the hybrid cell population, in vitro or in vivo, these are found to have lost the ability to produce an extracellular matrix.
Crosses between a sarcoma of fibroblastic origin and diploid lymphocytes

In all the crosses so far discussed, the non-malignant partner was a cell of fibroblastic type, and the malignant partner a cell of some other histogenetic origin. It was of interest to examine a mirror-image case: suppression of the tumorigenicity of a malignant cell of fibroblastic origin by fusion with a non-fibroblastic diploid cell. A9HT is a highly malignant sarcoma obtained by passage through a syngeneic mouse of a cell line originally derived from a single C3H mouse fibroblast (Wiener et al. 1973). In syngeneic, sublethally irradiated, new-born mice, it produces progressive tumours in 90% or more of recipients with inocula of $2 \times 10^5$ to $5 \times 10^5$ cells/mouse. When fused with diploid C57 Black lymphocytes, some hybrid clones can be isolated that are much less tumorigenic, giving take incidences of less than 10% under comparable conditions. A9HT × C57 Black diploid lymphocyte clone 3 is a highly tumorigenic hybrid clone that produces rapidly growing progressive tumours in nude mice essentially without lag. A9HT × C57 Black diploid lymphocyte clone 7, under the same conditions, produces nodules that remain stationary or regress over the 15-day period of observation. A histological examination was made of the lesions produced in the flanks of nude mice by the injection of A9HT cells, and this was compared with the lesions produced by the injection of the two A9HT × C57 Black diploid lymphocyte hybrid clones. A9HT injected into the flanks of nude mice produced rapidly growing undifferentiated sarcomas in which the cells were closely apposed and the cell membranes difficult to discern. No Van Gieson-stainable material separated the cells. The malignant A9HT × C57 Black diploid lymphocyte clone 3 produced progressive tumours similar in growth rate and in morphology to the A9HT tumours. But the A9HT × C57 Black diploid lymphocyte clone 7, in which growth in vivo was inhibited, produced non-progressive nodules which, at 10 and 15 days, showed a necrotic centre surrounded by cells aligned in a fibroblastic manner and separated by extracellular matrix that stained pink with Van Gieson's reagent. In one animal, a late progressive tumour arose 22 days after injection of the A9HT × diploid lymphocyte clone 7 cells. The lesion showed the original inoculum still present as a nodule with a necrotic centre surrounded by fibroblastic cells separated by collagenous extracellular matrix; but, at the periphery of the nodule, a secondary growth had appeared, composed of cells resembling the A9HT tumour in morphology, devoid of detectable extracellular matrix and having numerous mitoses. Clearly a segregant tumour. In these A9HT crosses, suppression of progressive growth is thus again associated with the production of a collagenous extracellular matrix. However, the results obtained with these crosses have a special interest because the diploid lymphocyte is itself unable to produce such a matrix. Nonetheless, it is able to restore this capacity to the defective A9HT cell. This indicates that the defect in the A9HT cell must involve a mechanism that is not specific for this particular form of differentiation, but one that is shared by both fibroblast and lymphocyte.
DISCUSSION

The observations described make it clear that, in the case of hybrids of fibroblastic lineage, only those that fail to produce a collagenous extracellular matrix grow progressively in the animal; cell multiplication in vivo ceases when the ability to produce this matrix is restored or imposed. Since the elaboration of a collagenous extracellular matrix in vivo is a manifestation of terminal differentiation in the fibroblast, the present findings provide us with another striking example of differentiation associated with cessation of cell multiplication. In hybrid cells this association is not restricted to fibroblastic differentiation. Peehl & Stanbridge (1982) have shown in crosses between malignant HeLa cells and diploid human keratinocytes that cell multiplication in vivo is suppressed, or at least greatly reduced, in those hybrids in which squamous differentiation and keratinization take place. It is, of course, a commonplace of tumour pathology that malignant tumours, for example squamous carcinoma, teratocarcinoma, osteosarcoma and fibrosarcoma, may generate sub-populations of terminally differentiated cells that are no longer capable of progressive multiplication. In the present context the case of fibrosarcoma is particularly interesting: the rate of growth of these tumours is inversely correlated with the amount of extracellular matrix that they produce, and in tumours where there is focal production of extracellular matrix, mitoses are found only in those areas where there is little or no matrix. (This may also be true for other tumours in which an extracellular matrix is produced, although the question does not appear to have been systematically examined.) But the studies on hybrid cells link the terminal differentiation to the suppression of cell multiplication in a more direct and decisive way. The present studies effectively eliminate the possibility that the two processes are concomitant but unconnected; and since, in vivo, the onset of the terminal differentiation precedes the suppression of cell multiplication it is reasonable to conclude that the cells stop multiplying because they undergo differentiation, rather than the reverse. If this is so, it is worth considering the possibility that an essentially similar relationship may govern cell multiplication under physiological conditions. To paraphrase Isaac Newton, one could propose that in metazoa, once the fertilized egg has been induced to multiply, all its descendants will continue to multiply until the process of differentiation compels them to stop. Multiplication, limited by nutrient supply and essential co-factors, might thus be envisaged as the natural steady state, and cessation of multiplication a restriction imposed on the system; multiplication would be re-initiated in cells in which it has been arrested when this restriction is removed. If this is so, the fundamental question in tumour biology becomes not what causes the cells to continue to multiply, but why the process of differentiation that would normally put a stop to their multiplication fails to occur. The general idea that malignancy might be a 'disease of differentiation' is, of course, not novel (Markert, 1968), and there are a number of cases where specific defects in the process of terminal differentiation have been defined in malignant cells (for review, see Jacob, 1983); but the model now being proposed envisages a central causative role for these defects in the generation of the malignant state and predicts that such defects will be found in all malignant cells.
This scheme of things may also offer a solution to what is probably the principal dilemma besetting current theory about the mode of action of oncogenes: how a score of different genes producing a range of proteins, some mutated, some not, of widely different sizes, shapes, solubilities, locations, binding affinities and, where determined, enzymic activities, all produce the same end result, 'transformation' \textit{in vitro} and, in some cases, progressive multiplication \textit{in vivo}. Much effort is at present being devoted to exploring the possibility that the oncogene product, either because it is structurally abnormal, or overproduced, or produced at an inappropriate time, stimulates cell multiplication. It may, however, be that these abnormal or inappropriate gene products act not by stimulating cell multiplication, but by impeding, in different ways, and perhaps at different points, the normal process of terminal differentiation. The progressive multiplication \textit{in vivo} and the morphological changes observed \textit{in vitro} are then to be seen as consequences of this impairment. There is experimental evidence that may be interpreted as lending support to this view. The present experiments show that transformation of NIH 3T3 cells by the H-ras oncogene renders them incapable of synthesizing collagenous extracellular matrix \textit{in vivo}. A similar effect has been observed for several different oncogenes \textit{in vitro}: transformation of fibroblastic cells by Rous sarcoma virus (Arbogast \textit{et al.} 1977; Vaherie \textit{et al.} 1978), by Simian virus 40 (Krieg \textit{et al.} 1980; Trüeb, Lewis & Carter, 1985) or by transfection with ras or mos oncogenes (Liau, Yamada & de Crombrugghe, 1985; Schmidt, Setoyama & de Crombrugghe, 1985) produces in all cases a severe impairment of the production of one or more of the components of the extracellular matrix. Again, the effect is not limited to fibroblastic differentiation. Yoakum \textit{et al.} (1985) have shown that transfection of bronchial epithelial cells by the H-ras oncogene renders them incapable of undergoing squamous differentiation; and Falcone, Tato & Alema (1985) have shown that several different oncogenes may similarly impair the differentiation of myogenic cells. The present model predicts that if the impairment of terminal differentiation is severe enough and stably inherited in the transfected cells, progressive multiplication \textit{in vivo} would follow. This prediction is, of course, open to experimental test. It seems, in any case, more plausible to suppose that abnormal proteins, or proteins produced at inappropriate times, or in inappropriate concentrations, act to impair cellular functions rather than to enhance them.

Finally, the present experiments shed some further light on the dominance relationships of the lesions determining malignancy. While 'dominance' and 'recessiveness' in the Mendelian sense are rather simplistic terms to describe what is going on in hybrid somatic cells, except as an operational description, it is now clear that if the normal cell with which the malignant cell is fused can impose a pattern of terminal differentiation on the hybrid, then malignancy, whether associated with the activity of a known oncogene or not, will be overridden; but if, as a result of chromosome loss or any other event, the normal cell cannot impose terminal differentiation, then malignancy will persist. It should, however, be noted that full terminal differentiation may rarely be achieved by cell lines \textit{in vitro}. Peehl & Stanbridge (1982) have shown that, in hybrids between HeLa cells and diploid
keratinocytes, squamous differentiation and keratinization go to completion only after the cells have been injected into the animal. In the case of fibroblastic cells, production of collagen and other components of the extracellular matrix does not bring cell multiplication to a halt in vitro until the cells have grown to confluence, and not even then if fresh medium is constantly perfused through the system (Kruse & Miedema, 1965). Apart from the fact that in vitro the cells are completely embedded in a collagenous extracellular matrix, whereas this is not the case in vitro, it is also possible that there are qualitative differences in what is produced in the two situations. Preliminary immunohistological tests with antisera directed against collagen type I and collagen type IV indicate that the defect in the malignant cells involves the production of collagen type I. Collagen type IV is present at tissue boundaries formed within and around the tumour. A systematic immunohistological examination is at present being undertaken with antisera directed against each of the major components of the extracellular matrix.

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