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Features Articles

Are Tumours Immortal?

Alasdair C. Stamps, Barry A. Gusterson and Michael J. O'Hare

INTRODUCTION

NORMAL MAMMALIAN CELLS in culture exhibit a finite proliferative lifespan before entering a state of irreversible and degenerative growth arrest known as senescence [1,2]. Occasionally, however, a cell escapes from senescence, acquiring apparently unlimited proliferative capacity and giving rise to what is termed an immortal cell line. The widely held notion that all tumours are immortal *in vivo* cannot, however, be rigorously tested by culture. Not only are many tumour cell types difficult to propagate *in vitro*, but the system itself may select for immortalising events occurring after introduction into culture. Thus, whether the equivalent of *in vitro* immortalisation of normal cells is an obligate event in multistep oncogenesis [3–5] cannot be directly answered in this manner. An understanding of the molecular mechanism of immortalisation of normal human cells *in vitro* will supply a genetic definition which can then be tested in the context of tumorigenesis.

SENESCENCE AND GROWTH CONTROL IN NORMAL CELLS

There is good evidence that *in vitro* senescence results from the expression of a biological programme that also operates *in vivo*. Correlations of declining cellular proliferation with age have been made in various tissues in mice [6] and in human epidermis [7]. Phenotypically senescent fibroblasts are also found in ageing hamsters [8]. Serially transplanted normal murine mammary glands exhibit a finite proliferative capacity, as do bone marrow stem cells [9]. The division potential of an individual culture of primary cells appears to be fixed and independent of their chronological age; thus, cells may be maintained in a quiescent, i.e. non-dividing, state for some time and then induced to divide without altering the final division number at senescence [10, 11]. Comparative studies of cell structural protein expression and hormonal responsiveness of cells *in vitro* with ageing tissues *in vivo* have identified a number of morphological and biochemical changes that are common to the two situations [12–16], further supporting the view that senescence *in vitro* reflects an *in vivo* phenomenon. Several groups have also demonstrated an inverse relationship between donor age and proliferative potential *in vitro* [17–20].

THE GENETIC BASIS OF CELLULAR MORTALITY

A number of lines of evidence point to a genetic basis for the mortal phenotype of normal cells. Somatic cell hybrids between primary and immortal cells, or between senescent and "young" fibroblasts, predominantly senesce [21, 22]. Transfer of human chromosome fragment 1q23–25 to immortal hamster fibroblasts induces senescent growth arrest [23, 24]. Thus, senescent potential is dominant over immortality. Cell fusions between disparate immortal human cell lines have shown that there are four

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complementation groups for senescence, indicating that the mortal phenotype may be directed by a small set of genes [25]. Recent microcell fusion experiments indicate that in one of these complementation groups, the lost gene(s) may reside on human chromosome 4 [26]. Quantitative studies of immortalisation frequency have given a figure close to 1 per 10^7 cells for human diploid fibroblasts infected by Simian Virus 40 ([27] and see below). This is consistent with immortalisation as the consequence of an event rare enough to be a genetic mutation [27, 28].

ESCAPE FROM SENESCENCE: EXTENDED GROWTH AND IMMORTALISATION

An immortal cell population theoretically has an infinite division capacity. This is obviously an impractical definition, so it is important to establish a reliable criterion by which to define immortalisation. There is an increasing tendency to ascribe immortality to cells exhibiting extended growth in short-term tissue culture experiments, although some authors regard cell lines as immortal only after 100 population doublings postsenescence [25], and others at 150 doublings postexplantation [27]. We suggest that 200 doublings after introduction into culture is a confident measure of human cell immortalisation, as it exceeds by a comfortable margin the maximum number of divisions that has been reported for normal human cells *in vitro*, i.e. 150 population doublings in neonatal keratinocytes [29] and is achievable in cell culture experiments within a realistic timescale (6–9 months).

There are distinct species-specific differences in observed rates of immortalisation or "establishment" of normal cells. Establishment of primary mouse cells in culture is a frequent and reproducible phenomenon, at around one immortalisation event per 10^5 cells [30]. This may be increased by the application of chemical carcinogens or the transfection of oncogenes [31]. The DNA tumour virus oncogenes, typically Simian Virus 40 large T antigen (T-Ag) [32], Human Papillomavirus (HPV) E6 and E7 genes [33] and Adenovirus E1 genes [34], are highly efficient promoters of rodent cell immortalisation and transformation. Rat and hamster cells spontaneously immortalise somewhat less readily, the latter showing evidence of a distinct senescent phase, while rabbit fibroblasts rarely and human cells almost never spontaneously immortalise *in vitro* [2]. Very rarely, a clone of immortal cells may arise apparently spontaneously from a long-term culture of normal human cells [35, 36], indicating an establishment frequency of less than one per 10^9 cells [37]. Studies of spontaneous human cell immortalisation are, therefore, virtually impossible. Interestingly, cells from at least some individuals with a familial cancer syndrome have a higher establishment frequency *in vitro*, although even this does not exceed one per 10^6–10^7 cells [38]. Treatment of human cells with chemical carcinogens does not result in a detectably higher frequency of immortalisation [39, 40], nor does transfection of single cellular oncogenes [41, 42]. Immortalisation of human cells at a low but predictable frequency does, however, occur after the stable transfection of oncogenes encoded by the DNA tumour viruses [43–45]. The frequency of human cell immortalisation obtained using these genes is still only around 1 per 10^7 cells. This does, however, provide a reproducible system for examining the mechanisms by which senescence occurs, enabling the study of immortalisation as a single genetic event in the absence of a significant background of other, spontaneous immortalising events such as occur in rodent cell culture.

HUMAN CELL IMMORTALISATION USING SV40 LARGE T-ANTIGEN

Extensive characterisation, the availability of stringent temperature-sensitive mutants and the encoding of all the necessary functions within one gene make SV40 T-Ag the agent of choice for these experiments. Normal human fibroblasts or epithelial cells transfected with the SV40 genome or its T-Ag gene have an extended growth span of 20–30 population doublings compared with untransfected cells [46, 47]. They then enter a "crisis" period, which may last from weeks to months, during which cell numbers become static then gradually decline, with an increasing appearance of gross karyotypic abnormalities and apparently failed mitoses [48, 49]. From these dying cultures occasionally arise clones with apparently infinite division potential. If wild-type SV40 is used, the emergent clones display progressive loss of differentiative capability [50], probably relating to their karyological instability [48, 51]. This instability, which affects cells both before and after immortalisation, appears to be due to repeated amplification and chromosomal integration of the SV40 genome which undergoes continual extrachromosomal replication in human cells both before and after crisis. These progressive changes have the effect of masking the primary immortalisation events with unrelated mutations. This problem has been avoided in recent studies by the use of a replication-defective recombinant amphotropic retroviral vector system [52] containing the SV40 T-Ag gene [53]. Cells infected by the retrovirus express T-Ag from a stably integrated provirus. They have an extended lifespan and immortalise at a low but predictable frequency as with wild-type SV40. If a suitable temperature-sensitive mutant of T-Ag, e.g. tsA58 [54] is substituted, the immortal lines derived are temperature-dependent for growth, arresting at around 39°C [55]. A construct of this type has been used in our laboratories to produce a panel of conditionally immortal cell lines from normal breast epithelial cells. These lines have the distinction of being stable, in terms of both their phenotypic and molecular markers, throughout extensive passage and recloning. Systems such as these are, therefore, well suited to the study of the molecular basis of cellular immortalisation.

THE ROLE OF T-Ag IN HUMAN CELL IMMORTALISATION

Despite their extended growth phase, human cells expressing T-Ag enter crisis after a proliferative lifespan inversely proportional to donor age at transfection [48]. Experiments using temperature-sensitive mutants or constructs expressing T-Ag from steroid-inducible promoters have shown that in the absence of fully functional T-Ag, both cells in the precrisis extended growth phase and postcrisis immortal cell lines arrest in the G1 phase of the cell cycle and gradually decline in viability [46, 55–58]. These findings indicate that the capability to senesce is retained by these cells but is somehow bypassed or overridden by functional T-Ag. Observed rates of immortalisation indicate, however, that this feature of T-Ag expression is not in itself sufficient to confer infinite growth potential upon human cells, and suggest that a second event is required.

MODELS OF IMMORTALISATION

Loss of mitogen response: the one-step model

Stein [47] has suggested that normal cells reach senescence due to a gradual decline to zero of mitogen responsiveness which may be overcome by the ability of SV40 T-Ag to initiate DNA synthesis [43]. Senescence is then delayed by the mitogenic

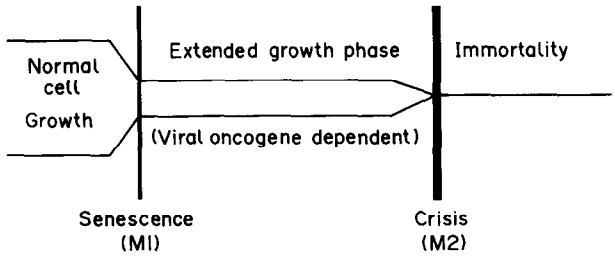

Fig. 1. The M1–M2 model of cellular immortalisation (based on Wright *et al.*, 1989 [59]).

action of T-Ag and manifests itself later as crisis in which programmes directing cells towards viable quiescence cause mitotic failure, implying that senescence (i.e. growth arrest in normal cultures) and crisis (i.e. growth arrest in cells expressing T-Ag) are caused by the same mechanism. This model would predict the re-expression of mitogen responsiveness to allow escape from crisis and thus T-Ag-independent growth of derived cells: as such, it is at variance with the effects of conditionally expressed T-Ag described above.

M1 and M2: the two-step model

Wright *et al.* [59] have proposed a two-stage model for human cell immortalisation (Fig. 1). Normal senescence is described as "mortality stage 1" (M1) and T-Ag-induced crisis as "mortality stage 2" (M2). The two main assumptions of the model are that M1 and M2 are caused by independent mechanisms and that the M1 mechanism is retained but suppressed by T-Ag in both extended growth phase and immortal cells. The model is based on the observations that: (a) large numbers of T-Ag transfected cells are able to traverse the M1 arrest point, but then become dependent upon T-Ag for growth; (b) escape from M2 occurs only at mutational frequency; and (c) immortal cells continue to be T-Ag-dependent. It predicts that in the absence of functional T-Ag, cells will enter normal senescence. Our laboratory and others have shown that immortalised and extended growth phase cells expressing temperature sensitive T-Ag remain viable after G1 arrest at the non-permissive temperature and may be rescued up to several weeks later by shift to the permissive temperature [55, 58]. These cells, therefore, behave as senescent cells, and in the absence of a known universal marker of senescence, this is the strongest evidence for the covert persistence of M1 in T-Ag-derived lines. As there are no differences in behaviour between extended growth phase and immortal cells in this respect, it would also appear that immortal cells are unique in that they are no longer able to express independent factors causing M2 crisis.

MECHANISMS OF EXTENDED GROWTH AND IMMORTALISATION

Models of senescence and immortalisation are necessarily speculative due to the paucity of accumulated data in this field. Recently, however, advances in our understanding of the interaction of T-Ag with cellular factors have suggested mechanisms by which it might promote cell division.

p53 and pRB

The "tumour suppressor" genes, p53 and pRb, are so named because of their ability to induce growth arrest when expressed in immortal cell lines [60–62]. The p53 and pRb proteins are bound *in vivo* by the tumour virus oncoproteins [63–68], and the absence of either of these viral binding activities is associated with loss of immortalisation-promoting activity [69–72]. Moreover, mutants of T-Ag which are defective in p53 binding complement those defective in pRb binding for growth of conditionally immortalised cells [70]. The tsA58 temperature-sensitive mutant of T-Ag, at the non-permissive temperature fails to bind p53 [71] and binds pRb with lower affinity [72]. These data suggest a role for p53 and pRb in cell cycle arrest which is blocked by binding to T-Ag.

The cell cycle control function of pRb is dependent upon its phosphorylation status: underphosphorylation of this protein appears to present a block to S phase entry [73], and pRb is underphosphorylated in senescent fibroblasts [74, 75]. T-Ag binds preferentially to underphosphorylated pRb [76], suggesting that this may overcome the block to progression through S phase. p53 competes with DNA polymerase α for binding to T-Ag [77]. T-Ag may, therefore, substitute for an as yet undefined cellular factor to which these two proteins competitively bind. This factor may be involved in the initiation of DNA synthesis as T-Ag has been shown to be associated with the cellular DNA replication complex and contains a helicase activity [78].

p53 is the most frequently mutated gene so far analysed in cancers [79], and human cells with germ-line p53 mutations have an increased spontaneous immortalisation frequency *in vitro* [38]. The Rb gene is also frequently mutated in certain types of cancer [80]. In HPV-positive cell lines derived from anal carcinomas, no p53 or Rb gene mutations were found, whereas papillomavirus-negative lines derived from similar tumours all had p53 mutations [81]. Viral oncoproteins may, therefore, override the M1 mechanism by binding to and inactivating p53, pRb and perhaps other cellular proteins, effectively substituting for mutations in these genes in immortal cells. However, many immortal cell lines exist in which normal p53 and pRb are expressed. Thus, although p53 and pRb probably have a role in M1 senescence at a control point in the processes leading to permanent cell cycle arrest, other tumour suppressors (reviewed in [82]) could also be involved.

DNA synthesis inhibitors

Factors inhibiting DNA synthesis are possible candidates for effectors of mortality. Short-term inhibition of protein synthesis delays the onset of fusion-induced growth arrest in hybrids of normal and senescent cells [83], and microinjection of mRNA from senescent cells inhibits S phase entry in proliferating cells [84], indicating that the onset of senescence is mediated by protein factors. Further experiments on the complementation groups described by Pereira-Smith and Smith [25] demonstrated that an unidentified membrane-bound protein isolated from cells of one complementation group inhibited DNA synthesis in normal human cells and in cells of one other complementation group [85]. An evolutionarily conserved protein, prohibitin, prevents DNA synthesis in normal and tumour cells [86]. Another protein associated with non-proliferative states is statin [87], also known as pSEN [88], which contains sequences identical to ribosomal elongation factor 1α along part of its length and which is found in a variety of non-dividing cell types. It has yet to be shown whether any of these proteins is associated exclusively with senescent cells, i.e. whether they are part of a general mechanism for preventing cell growth or specifically brought into play at senescence. There is no evidence as yet for mutations of these or other genes involved in the regulation of DNA synthesis (e.g. the cyclins or cdc2-related genes) being associated with immortality.

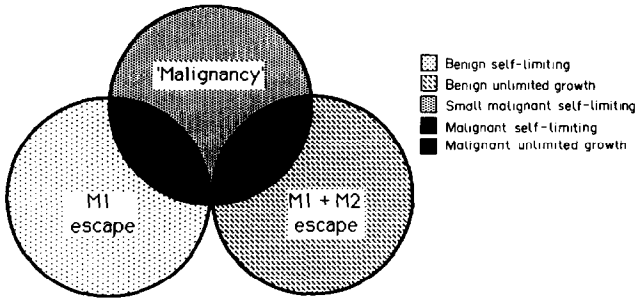

Fig. 2. Possible consequences of the M1–M2 model for human tumours.

IMMORTALITY AND CANCER

Senescence may have evolved as a mechanism to protect the organism against mutationally acquired clonal hyperproliferation in somatic tissues. The sequence of genetic events resulting in the acquisition of tumour phenotypes, such as failure to differentiate, exponential growth, invasiveness and metastasis, seems to be random [5]. Immortalisation is not necessarily a prerequisite for tumorigenicity, as *in vitro* precrisis SV40-infected cells can form tumours in nude mice [51]. Thus, cellular tumour phenotypes are expressed completely independently of immortalisation status. The time required *in vivo* to accumulate the minimum number of events resulting in malignancy [5, 89] is considerable and may bring cells close to the normal limit of their proliferative lifespan, in which case senescence would intervene before the malignant cells could divide into a population size sufficient to threaten normal organ function. Since normal senescence (M1) [59] appears to be an *in vivo* phenomenon, it seems reasonable to postulate that the equivalent of the *in vitro* M2 mechanism also operates *in vivo* as a second line of defence against inappropriate cell growth, particularly as some of the viruses which cause M1 traverse are associated with naturally occurring cancers. If a single cell acquired the ability to traverse M1, M2 would follow after between ~20 and 30 additional population doublings [46, 47]. Assuming 100% cell survival and exponential growth, this would result in 10^6–10^8 cells, occupying a volume of about 2 mm^3–0.2 cm^3, when regression would supervene as a result of M2 crisis. This could manifest itself as a dysplasia, a small adenoma or carcinoma *in situ*, depending on whether malignancy-conferring genetic events had also occurred. Some experimental evidence for this has been shown in colonic tubular adenoma cells which senesce in culture after an initial period of growth [90]. Infection by, for instance, HPV in keratinocytes or EBV in lymphocytes, may result in M1 escape for a relatively large number of cells, but the imposition of factors such as terminal differentiation would considerably reduce the number of cells surviving to M2. The monoclonality of most cancers [91, 92] argues against the possibility of large numbers of cells escaping M1 to form a tumour, and for the existence of a real M2 barrier *in vivo*. Only if M2 traverse occurred would a truly immortal tumour result with benign or malignant characteristics conferred by prior or subsequent independent genetic events.

According to this model, tumours found in the human body should range from a theoretical malignant but mortal single cell at one extreme, to a benign, differentiating and relatively organised but immortal tumour which grows to an enormous size without killing the patient, with the majority of "cancers" falling between these points, representing a subset which has acquired both immortality and malignancy (Fig. 2). This potential spectrum of independently acquired growth and malignancy-associated characteristics raises the possibility that some diagnosed cancers of small size (e.g. some *in situ* carcinomas) may not, in fact, be immortal and might not, in the absence of medical intervention, progress to a life-threatening tumour. This is an important "grey" area in terms of both diagnosis and prognosis which might be illuminated by the discovery of factors which cause the mortal phenotype.

The notion that all tumour cells are immortal is, nevertheless, widely accepted, to the extent that many researchers consider it a definition of cancer and believe as a corollary that the establishment of tumour cell lines is a straightforward exercise. In practice, however, success rates vary greatly from one tumour cell type to another, ranging from 50% or more of malignant melanomas to 1% or less of breast cancers [93]. This raises the question of whether this is simply due to technical difficulty of propagating some tumour cell types or whether in some tumours the majority of cells are not, in fact, immortal. As some tumour cells may be even more dependent on the presence of specific growth factors than normal cells, a very powerful selective pressure may exist in culture for genetic events which confer independent growth *in vitro*. By the time proliferating tumour cells have been established, it may be difficult to distinguish immortalisation events which have occurred in culture from events which occurred in the original tumour. For this reason, immortalisation needs to be defined by other, molecular criteria before statements regarding the immortal status of tumour cells may be made.

FUTURE DIRECTIONS

The study of senescence and immortalisation of normal cells in itself presents a major challenge. Senescence occurs after an apparently preset number of mitoses. How do cells "count" these divisions?

Harley *et al.* [94] have described evidence that the length of human chromosomal telomeric repeat sequences declines in proportion to cell division number. Loss of telomeres in yeast results in chromosomal instability and loss of viability. These observations led to the proposal that cellular senescence may result from the eventual loss of telomeres and corresponding chromosomal disintegration [95]. However, mouse telomere length does not decrease upon cell division [96] and in Drosophila, telomere loss does not lead to chromosomal instability [97]. Thus, if there is a mechanism of senescence involving chromosomal telomeres in eukaryotes, it does not operate in the same way in all species and may not operate at all in some.

So-called stochastic ageing theories claim the loss of proliferative potential with age to be due to accumulating randon genomic damage in somatic cells. Holliday [37] has proposed that senescence is the result of epigenetic alterations caused by random demethylation of transcriptional promoters.

These theories do not account for observations of extended growth potential in cells expressing T-Ag or the establishment of stable, immortalised cell lines from single cells in crisis. The work of Pereira-Smith and Smith [25] and of Wright *et al.* [27] strongly supports a single event hypothesis to explain the latter phenomenon. As senescence may be induced in growing cells via factors apparently exclusively expressed in senescent cells [21, 22, 84], the existence of dominant genes for the mortal phenotype is implied, suggesting that senescence is, in essence a "differentiative" event [98]. Studies employing genetic subtraction should lead to the isolation of these genes.

Subtractive cDNA analysis has been used successfully in other differentiating systems, notably in the discovery of the

other differentiating systems, notably in the discovery of the myoD myogenesis factor [99], but subtractions between normal and immortal human cells have as yet yielded little of interest [100,101]. This may be because the comparisons were made between presenescent normal cells, a tumour-derived cell line and a separately derived tumorigenic cell line, representing the most phenotypically divergent examples of a single cell type. A subtractive analysis between human cells at or near T-Ag-induced crisis and a panel of stably immortalised cell lines derived from the same cells, or between members of different complementation groups within such a panel, would yield cDNA libraries representative of changes occurring upon immortalisation which would be more amenable to analysis. The discovery of genes directing the senescent blockade could provide probes with potential prognostic implications with which to examine cancers, as well as precancerous conditions and predispositions to subsequent cancer development.

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