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Control of Fibroblast Senescence and Activation of Programmed Cell Death

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Abstract We have characterized a nuclear phosphoprotein of 57 kda, statin, found only in nonproliferating cells of both quiescent and senescent natures. Emerging results suggest that statin may function as a sequester to block the early G_1 phase phosphorylation for the RB protein. A second protein, terminin, undergoes senescence-specific posttranslational modification from 90 to 60 kda, and further death-specific conversion from 60 to 30 kda. We also found that apoptotic mouse 3T3 fibroblasts express c-*fos*, c-*myc*, c-*jun*, and *cdc2*, as well as the upregulation of RB phosphorylation and BrdU incorporation, just before final DNA fragmentation and death. It seems that en route to death, cells re-enter the cell-cycle traverse and experience early G_1 and part of S Phase; however, this cycling event is an abortive one. In contrast, senescent fibroblasts are resistant to the initiation of the death program, since they are unable to enter cell cycle traverse. Long-term serial passaging of normal human fibroblasts may be inadvertently selecting those, while termed as senescent, are also specialized survivors, and thus a good culture model to study both the control of permanent departure from cell cycle traverse and the mechanism underlying the survival or antideath cellular program.

Key words: replicative senescence, cell-cycle traverse, programmed cell death, apoptosis, oncogenes and anti-oncogenes

We take for granted many wonders governing our body's proper function. For example, how many cardiomyocytes are needed to generate optimal contractility for the heart? Similarly, how many neurons are needed to complete a single action of cognition or motion? Emerging knowledge suggests that the number and size of cells in a given tissue are probably controlled by a predefined genetic program. The total number of cells in any given tissue is in general determined by a predetermined program of action, commencing with the rapid growth phase to create sufficient cell mass, through the mature nongrowing and differentiated phase to fulfill the predestined function of the tissue. Cell numbers of most tissues are then defined by the simple logic of the accumulation of growing and nongrowing subpopulations.

Unfortunately, things are not as simple as they may seem. Integrated into the balance of growing and nongrowing populations are many factors, such as the time required for the growing cells to replicate themselves, and how long the nongrowing cells survive after their long span of differentiation. To complicate matters even more, every tissue seems to have its own developmental career of proliferation. For example, cardiomyocytes and neurons have completed most of their proliferative lifespan by the time of birth, while hemopoietic cells display continuing replicative activity in their stem cell population throughout life. Two key transitions govern the normal differentiation program: when and how growing cells should cease growing, and when and how excess nonproliferating cells should die to make room for the newcomers. Perturbation of the first transition may result in cancer; perturbation of the second transition, however, may result in a different type of abnormality, namely excess cell loss that tissues can ill afford. Growth factors, oncogenes and anti-oncogenes, mitogens, and so forth, or lack thereof, are thought to be the necessary tools nature provides to moderate the first transition.

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What then controls the second transition, or more precisely how and when is the death process activated in a cell? Might the presence of putative death and antideath genes be governing this process?

FIBROBLAST SENESCENCE

The postreproductive career of cultured human fibroblasts is defined by the loss of potential for further DNA synthesis, leading to failure of cell replication. This phenomenon of loss of proliferative capacity after a defined number of population doublings was described by Hayflick and Moorhead [1961] as the manifestation of aging at the cellular level; when cells reach the stage of replicative incompetence, they are considered to be senescent. The failure of senescent cells to synthesize DNA upon mitogenic stimulation seems to be due to the combined effect of the loss of proliferation-stimulating, and the initiation of nonproliferation-specific, gene expressions. To support this notion that cellular senescence is controlled by counteracting forces, reports on the gain or loss of gene expressions associated with aging are plentiful; highlights include the loss of c-*fos* proto-oncogene [Seshadri and Campisi, 1990], *cdc2* and cyclin A [Stein et al., 1991], as well as of telomere length [Harley et al., 1990], and the inability of the gain of RB protein phosphorylation and therefore the retaining of its anti-oncogene function [Stein et al., 1990], the gain of gene expressions such as prohibitin [McClung et al., 1989], *pSen* [Giordano and Foster, 1989], and so on. Our own work on the unique presence of terminin and statin in senescent fibroblasts [Wang, 1985, 1992; Wang and Tomaszewski, 1991], together with the above results, provides molecular markers to demarcate the phenotypes of in vitro aged cells.

DEFINING NONPROLIFERATING STATUS BY BIOCHEMICAL MARKERS

Cell hybrid studies [Rabinovitch and Norwood, 1980; Stein and Yanishevsky, 1981], as well as recent microcell fusion studies, have shown that the irreversible growth arrest in serially passaged and aged human fibroblasts is not only a dominant phenotype, but is also attributed to the functions of genes specifically localized in chromosome 1 [Sugawara et al., 1990] and 4 [Ning et al., 1991]. This work, along with that on the inhibition of DNA synthesis in young cell nuclei by microinjection of mRNA isolated from old cells [Lumpkin, 1986], has led to the suggestion that there are proteins uniquely expressed in nongrowing cells but not in their growing counterparts, and it is the function of their genes that eventually elicits the inhibition or prevention of DNA synthesis. In the attempt to identify these proteins, we have used the approach of developing specific monoclonal antibodies which recognize protein(s) in nongrowing cells; this attempt has led us in the last few years to characterize a nuclear protein, statin, which is uniquely present in nongrowing cells, whether quiescent or senescent (reversibly or irreversibly growth-arrested, respectively). The presence of statin has been characterized as a marker for those cells that have ceased proliferation and entered the nonreplicative state in both cultured cells [Wang and Lin, 1986] and tissues [Wang and Krueger, 1985]; the quantitation of statin-positivity has been used as a negative index for evaluation of tumor malignancy [Tsanaclis et al., 1991]. Recent results have shown that statin is a 57-kda nonglycosylated protein, phosphorylated by an associated serine/threonine kinase of 45-kda, which is coprecipitated with statin [Lee et al., 1992]; a third protein that coprecipitates with them has been characterized as the unphosphorylated form of retinoblastoma (RB) protein. Emerging from this picture is the idea that statin may function as the sequester or blocker for the kinase action of the p45 serine/threonine kinase; in nonproliferating cells, this sequestering action may keep the RB protein from direct contact with the kinase, so that it remains unphosphorylated. Since RB needs to be in the unphosphorylated state to be antiproliferatively functional, the prevention of kinase action by a sequester such as statin provides an efficient way to maintain a specific, complex biochemical state. We are at this point attempting to confirm the sequestration action of statin by addressing the following questions: (1) can p45 kinase phosphorylate RB when it is separated from statin?, (2) if so, does the p45 kinase affect specifically the first wave of RB phosphorylation sites, and how does this differ from the p34 *cdc2* kinase action on RB? Ultimately, the precise function of statin in blocking RB phosphorylation, and its implication to replication arrest, needs to be addressed by the use of molecular tools such as antisense RNA.

POSSIBLE MOLECULAR MECHANISMS FOR THE CONTROL OF NONPROLIFERATING CELL-SPECIFIC GENE EXPRESSION

Our own work on statin, and that of other laboratories, suggests that many nonproliferating cell-specific gene expressions are transcriptionally controlled. In other words, the presence of these proteins in nongrowing cells is not due to the modification of preexisting RNA message or polypeptides; rather, it is due to the lack of, or *inability to identify*, a translatable message in the growing cells. Based on our current knowledge, we can hypothesize that the absence of the gene expressions is due to either: (1) the absence of signal to elicit the expression, such as the absence of a growth factor or its successful chain reaction; or (2) the presence of a repressor which is activated in the presence of a growth factor, which in turn suppresses the expression of the unique nongrowing cell genes. The dominance of replicative senescence leads us to favor the latter possibility. For example, as shown in Figure 1A, adding growth factors to quiescent cells (those in reversible growth arrest) activates a common stimulator responsible for activation of a proto-oncogene such as c-*fos;* when this is expressed, it in turn initiates AP-1 trans-activation. At the same time, the same stimulator may activate a novel gene, statin-repressor (SR), which provides negative control of statin gene expression. In senescent cells, as depicted in Figure 1B, the lack of growth factor response or the interruption of the signal-transduction response suppresses this common stimulator. The cascade consequence will be the inability to express both c-*fos* and statin repressor; the subsequent indirect effect is the permanent expression of proteins such as statin. The same hypothetical model can be used to explain the molecular mechanism controlling other related nonproliferation-specific gene expressions. Of course, we realize that this model may be sub-

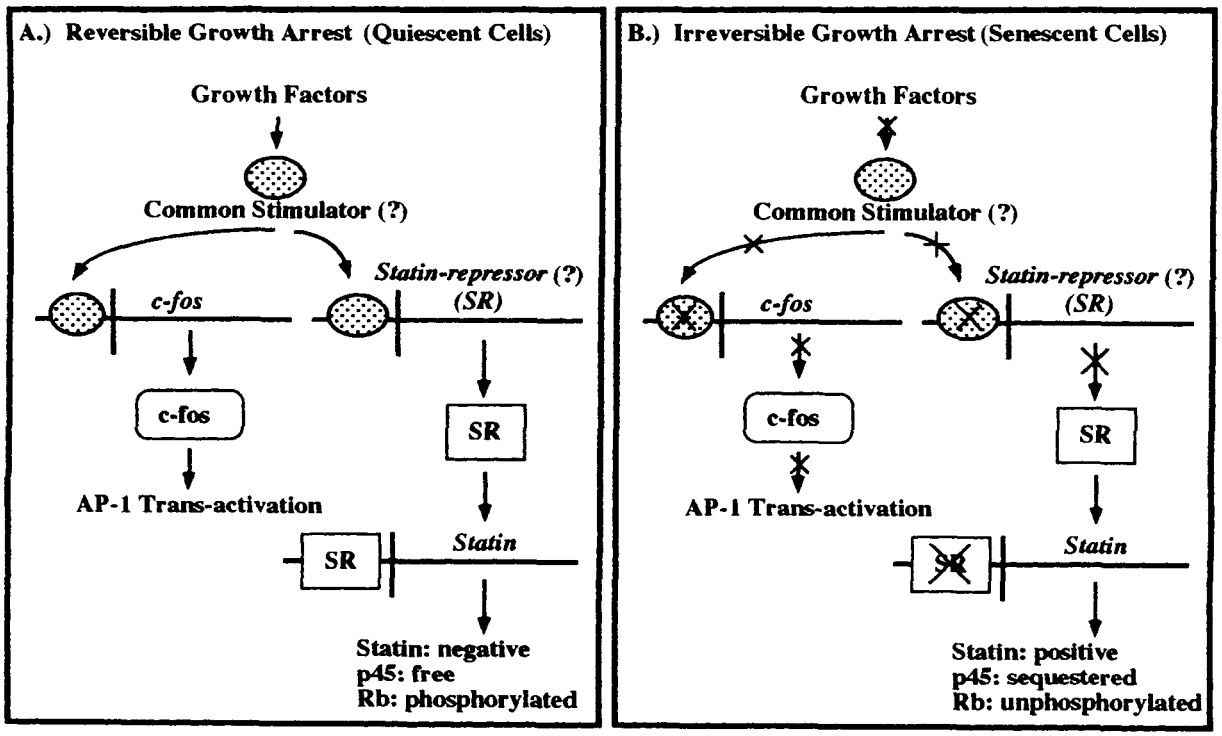

Fig. 1. Diagrammatic model illustrating a possible molecular mechanism governing the up or downregulation of statin expression in quiescent or senescent cells. **A:** Circumstance in which growth arrest is reversible by adding some growth factors back into the culture, which in turn activates a putative common stimulator, which may bind to the promoter region of c-*fos* as well as of the statin repressor (SR). The consequent effect of this action may be the repressive action of SR at the promoter region of statin, which then suppresses statin expression and releases the p45 kinase from its sequestering action; RB protein then becomes phosphorylated. **B:** Possible modulation of this hypothesis in the case of irreversible growth arrest in senescent cells, where the whole cascade of the presence of growth factors and the activations of the putative common stimulator, c-*fos,* and statin repressor are all turned off. The end result is the positive expression of statin, which then sequesters the p45 kinase and thus keeps the RB protein in the unphosphorylated state.

ject to considerable modification, and validating it depends on future work to identify the two key novel genes, namely the common growth factors or signal transduction-dependent common promoters, as well as novel genes such as statin repressor.

SENESCENCE- VERSUS QUIESCENCE-SPECIFIC PHENOTYPES

Clearly, the unique physiological landmark of fibroblast senescence is the fact that, aside from strenuous manipulation such as introduction of SV-40 T antigen, the ability of DNA synthesis is permanently shut off [Wright et al., 1989]. Our identification of statin, and others' identification of unique genes responsible for or associated with replication arrest, makes us realize that these observations are applicable to both quiescent and senescent fibroblasts. Therefore, there is the need to define the unique gene expressions pertaining only to senescent fibroblasts and not their quiescent counterparts; along this line, we have characterized a protein, terminin, whose presence in different molecular weight forms is dependent on distinct physiological states. In general, in young growing and nongrowing quiescent cells, terminin is present in a 90/87-kda form; in senescent cells, it is found in a 60/57-kda form. Histochemically, terminin antibody staining activity is detected only in the cytoplasm of senescent cells, in a granular distribution; Tp90 is not detectable histochemically. The positive staining index of terminin antibody in cultured normal fibroblasts is directly proportional to the cumulative population doubling level. Conveniently, one may use either the amount of terminin protein (Tp60/57) or the number of cells positive for terminin antibody staining as a quantitative index to indicate the life span remaining in any given in vitro culture of normal human fibroblasts.

By accident, we discovered that the few dying cells of the quiescent cultures show positive terminin antibody staining activity; this observation led us to investigate whether terminin positivity is also related to the programmed cell death program. Analysis of activation of programmed cell death in mouse 3T3 fibroblasts reveals that indeed terminin is detected; however, from its position on the SDS gel, the protein is in a 30-kda form. Therefore, the presence of terminin polypeptide in the three different molecular weight forms, 90, 60, and 30 kda, is highly dependent on the physiological status of the cell; Tp60 is found in senescent cells, while Tp30 is found in dying cells. As shown in Figure 2, the presence of Tp30 at 12–18 h after the initiation of programmed cell death marks the time point where commitment to apoptosis sets in; thereafter, addition of serum to the culture cannot rescue cells from DNA fragmentation and death. Other results have shown that there may be specific proteases involved in the production of the various terminin polypeptides. Therefore, one may suggest that there exist two different types of proteases, specific respectively to senescence and programmed cell death (apoptosis); how and when what processes activate these proteases in the two disparate physiological states, as well as their identities, become important questions for us to address. The terminin protein itself thus becomes a fishing rod for us to use to study these questions.

PROGRAMMED CELL DEATH AND CELL CYCLE TRAVERSE

Surprisingly, during our investigation of terminin presence we found that the dying cells display upregulation of expressions for proto-oncogenes such as c-*fos*, c-*myc*, c-*jun*, and *cdc2*, as well as of the conversion of RB from the unphosphorylated to the phosphorylated form. Furthermore, some dying cells exhibit BdrU labeling activity. These unexpected results sug-

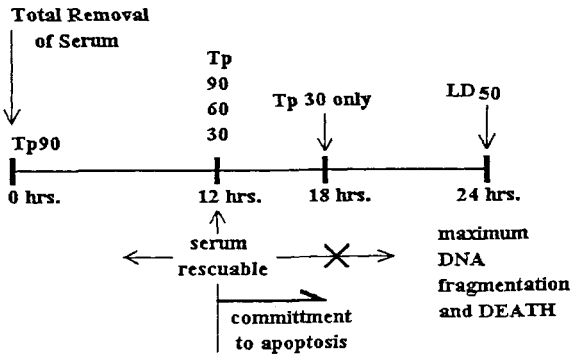

Fig. 2. Diagrammatic model illustrating the presence of terminin protein (Tp) in three different forms: 90, 60, and 30 kda. Tp90 is the only form observed at the initiation of programmed cell death (apoptosis) by removal of serum in mouse 3T3 fibroblasts. At 12 h after the initiation, all three forms are observed, Tp90, Tp60, and Tp30; at this time the process of apoptosis is still rescuable by adding serum back to the medium. After the 12-h timepoint, Tp30 becomes the predominant form and by 18 h, this is the only terminin polypeptide present; DNA fragmentation is most dramatic, ultimately resulting in the final event of death at 24 h.

gest that cells en route to apoptosis may undergo a departure from the quiescent state and even enter early G_1 phase, initiating some DNA synthesis activity before dying. As shown in Figure 3, the progress of programmed cell death (apoptosis) may include something like G_1 and early S-phase of the cell cycle traverse. We recognize that in this model we cannot yet rule out the possibility that the BrdU activity is due to DNA repair; however, our observation of the upregulation of PCNA, a DNA polymerase δ, an integral member of the DNA replication process, provides a bias against this notion. Nevertheless, our results and those of Buttyan's laboratory [Colombel et al., 1992] suggest that for apoptosis to occur, cells may need to leave the quiescent state and experience part of G_1 and an abortive S-phase before death finally occurs. A cautionary note to this suggestion is the ample evidence of many different pathways which programmed cell death can follow. Thus, the applicability of our model of reentrance of cell cycle traverse and abortive DNA synthesis may be limited to mouse 3T3 fibroblasts and castration-induced prostatic epithelial cells.

FIBROBLAST SENESCENCE, CELL CYCLE TRAVERSE, AND ANTI-PROGRAMMED CELL DEATH

During our investigation of the presence of Tp30 in dying 3T3 fibroblasts, we also attempted a similar approach to examine whether the terminin present in the 60 kda form could be converted to the 30 kda form in senescent fibroblasts. Surprisingly, we could not induce the activation of programmed cell death in cultures of old cells by the usual means, such as deprivation of serum or treatment with arabinoside C.

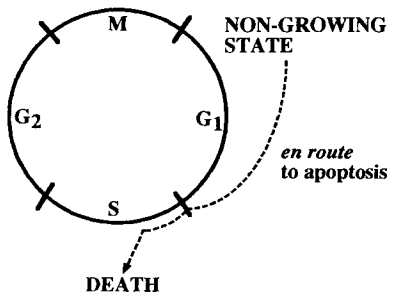

Fig. 3. The possible sharing of G_1 and entrance to S phase between normal cell cycle traverse (solid line) and programmed cell death (apoptosis—dashed line) pathways. For the apoptotic pathway starting from the quiescent state, the departure from the nongrowing state is followed by entrance into G_1 and subsequently S-phase, but with the abortive result of death.

It seems that senescent cells have an unusual survival capability and resist our attempts to induce apoptosis. Preliminary observation shows that senescent fibroblasts contain high levels of *bcl2* expression, while the immortalized mouse 3T3 fibroblasts exhibit undetectable or low levels of this protein. This observation, along with the repression of c-fos and RB phosphorylation, leads to viewing senescent cells from totally different angles. As shown in Figure 4, the requirement of early cell cycle genes, *cdc2* and RB phosphorylation, may have a dual purpose, with the major goal of successful DNA synthesis and completing the replicative cycle, while another minor path is an abortive traverse whose second half is occupied by the activation of death genes and the final apoptotic mortality of the cells. For senescent cells, the inability to return to cell cycle traverse also serves as a protection from programmed cell death, since both successful cell cycle traverse and death-associated apoptosis share at least in part a short segment of the beginning G_1 and early S phases. It then becomes clear, as shown in Figure 5, that senescent fibroblasts are those cells which can express neither growth-promoting genes, in order to proliferate, nor death genes, in order to enter the pathway leading to programmed cell death.

CELLULAR SENESCENCE REVISITED

A constant debate among those who work with phase III fibroblasts (according to the original Hayflick model) is whether these permanently growth-arrested cells indeed represent aging at the cellular level, or whether they are simply the terminally differentiated form of cultured fibroblasts [Goldstein, 1990; Peacocke and Campisi, 1991]. Lack of specific terminal differentiation markers to define specific differentiated stages of fibroblasts makes it almost impossible to reach a final verdict for this debate; the pendulum swings periodically among the various investigators, and the issue remains an elusive mystery in the back of everyone's mind. Nevertheless, over the years there has emerged a healthy altitude toward this mystery: the utilization of Hayflicks's phase III fibroblasts as an excellent cell model to study the control of cell proliferation and the permanent shut-off mechanism for this process. Whether senescent fibroblasts are terminally differentiated or not, they are still a good model to study how long-lived cells such as neurons and cardiomyocytes permanently turn off their replicative potentials, and

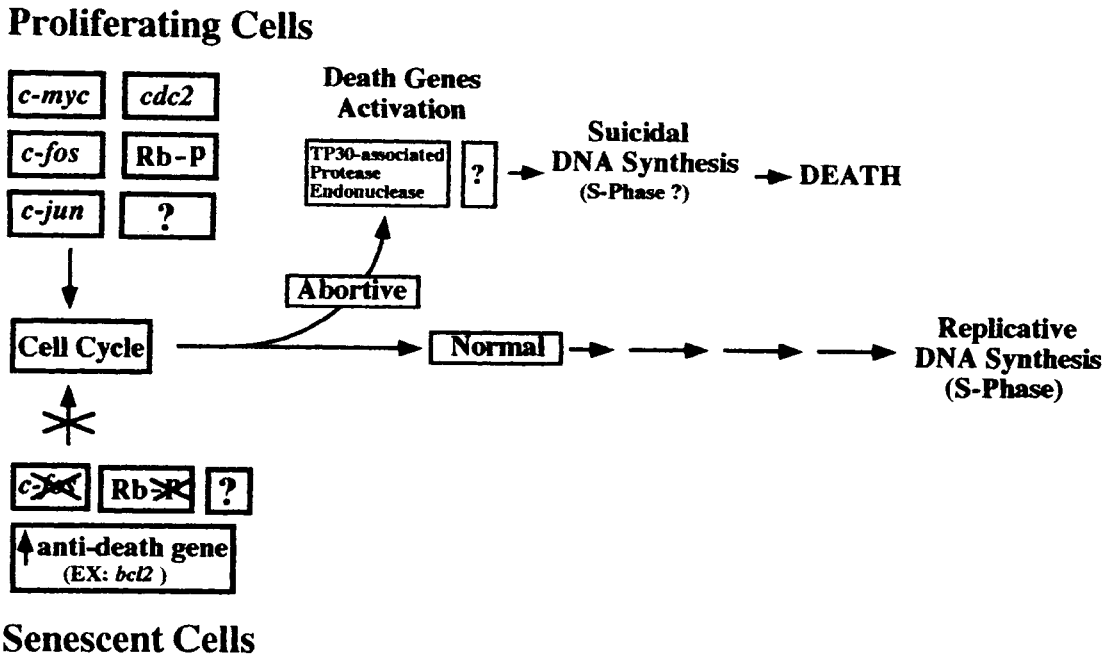

Fig. 4. Diagrammatic illustration suggesting possible pathways by which growing or nongrowing quiescent cells can experience cell cycle traverse under the molecular actions of c-*fos*, c-*myc*, c-*jun*, *cdc2*, phosphorylated RB, and other possible cell cycle control factors. The normal cell cycle traverse will lead to successful DNA synthesis in S-phase and completion of the replicative cycle. The detour from this pathway will lead to the abortive dead end with the activation of death genes, protease and endonuclease activity, appearance of terminin protein in the 30-kda form (Tp30), and the eventual suicidal DNA synthesis followed by death. For senescent cells, neither of the above pathways can be initiated because c-*fos* expression, RB phosphorylation, and other cell cycle gene expressions such as *cdc2* are repressed. The senescent cells' incapability to enter the above pathways may also be reinforced by the fact that antideath gene *bcl2* is upregulated, which prevents programmed cell death from becoming initiated.

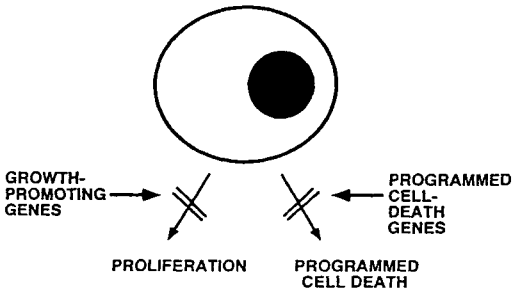

Fig. 5. Diagrammatic illustration describing the unique properties of senescent human fibroblasts, i.e., the inability to enter either the proliferation phase or programmed cell death, since apparently neither growth-promoting nor programmed cell death genes are effective.

yet remain functional and living throughout the organism's life span.

As discussed above, the senescent phenotype may be viewed as an antideath state. By a close examination of the process of obtaining cultures of senescent fibroblasts, one may begin to wonder whether, during the exercise of establishing primary cultures from skin biopsy and the subsequent serial passaging manipulation, we might indeed unknowingly introduce a selection process favoring survivors. The weaker, dying cells usually degenerate, become suspended in the culture medium, and are discarded when mother cultures are split into daughters; since most of us do not routinely examine those suspended dying cells, and focus our observation and manipulation on adherent cells, the process of serially passaging cells to obtain senescent cultured fibroblasts in itself becomes a stringent selection procedure for obtaining survivors, as illustrated in Figure 6. It then becomes logical, and even perhaps inevitable, that our attempt to induce programmed cell death in senescent fibroblasts was unsuccessful, because these cells are selected for the greatest potential to resist death. The eventual result of establishing such cell lines may be the selection of that subpopulation of cells in the original primary culture possessing a high level of antideath gene expressions; our preliminary observation of high levels of *bcl2* gene expression suggests that this possibility is likely. If one accepts the hypothesis that the senescent fibroblast phenotype is also an

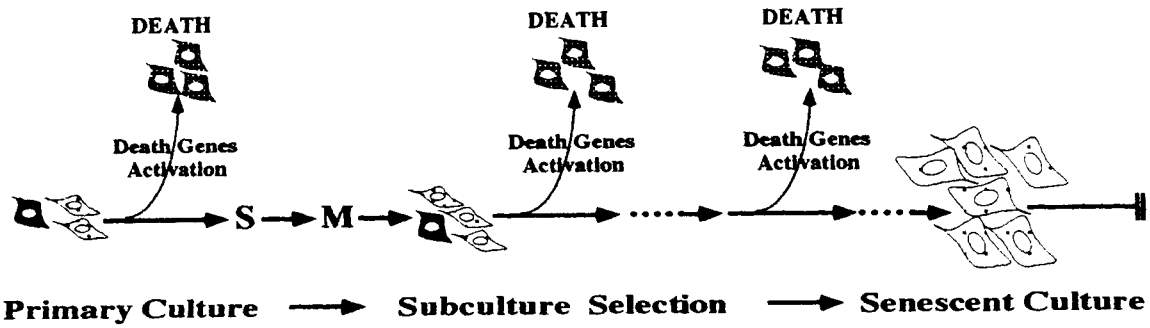

Fig. 6. Diagrammatic drawing illustrating possible mechanism of selection for death-resistant cells during the process of primary culturing and serial passaging, while attempting to establish senescent cultures. At first, many dead cells are observed in the primary skin biopsy culture; the survivors experience DNA synthesis (S) phase and mitosis (M), and eventually become a stabilized culture. However, during each passaging from mother to daughter culture, subculture selection allows the few dead cells to float into the suspension and become discarded. The long-term end result of this selection provides a senescent culture which may be viewed as the ultimate population surviving the selection process. Therefore, the whole process of establishing senescent fibroblast cultures could be viewed as selecting cells which are resistant to death.

antideath state, the usefulness of senescent fibroblasts as cells to search for antideath genes becomes obvious. We are therefore bestowed with the gift of an excellent model, namely Hayflick's phase III fibroblasts, as a framework allowing us the freedom and luxury to investigate and research the wonder of nature in controlling not only cell replication, but also programmed cell death. Both of these processes are vital to our understanding of mechanisms dictating the intriguing balance among growth, differentiation and death; the application to disease situations when such a balance is interrupted is obvious and significant. Whether we call permanently growth-arrested cultured fibroblasts senescent, terminally differentiated, or antideath is less significant than the knowledge and information to be gained when and if we focus our study on their underlying molecular orchestration. Future experiments studying in vitro cultured senescent fibroblasts will certainly unfold facts to substantiate our claim or contradict our hypothesis with new insight.

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