Loss of Transformed Phenotype upon Senescence of Rous Sarcoma Virus-Infected Chicken Neuroretinal Cells†

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Success in obtaining permanent Rous sarcoma virus-infected chicken cell lines has been limited because of a senescence phenomenon. We show that a diminished, transformed phenotype, followed by dramatic morphological changes, precedes senescence. These changes are associated with continued expression of pp60^{v-src}, as well as specific alterations in expression of two possible phosphorylated substrates of pp60^{v-src}.

Success in obtaining permanently established Rous sarcoma virus (RSV)-infected chicken cell lines (7, 10, 39) has often been hindered by the phenomenon of spontaneous degeneration and death in RSV-transformed chicken cell cultures (26). Thus, RSV immortalization of chicken cells has been described as an extremely rare event (39). In the case of embryonic chick neuroretinal cells transformed with RSV (23, 24), cells survive for approximately 15 passages and exhibit morphological and biochemical changes, such as inhibition of histogenesis (39) and loss of the neural surface marker N-CAM (2), as well as an increase in choline acetyltransferase activity (22). Thus, although the proto-oncogene counterpart of v-src is expressed in developing chicken neuroretina (34) and other neural tissues (5), the introduction of pp60^{v-src} into chicken neuroretinal cells leads to down-regulation of specific developmentally regulated genes (11) and inhibition of cellular differentiation (40). These effects are usually temporary, however, as RSV-transformed chicken neuroretinal cell cultures also succumb to senescence, a phenomenon which has not been widely studied *in vitro*.

To characterize this degenerative phenomenon, we have infected embryonic day 7 chicken neuroretinal cells with a temperature-sensitive Prague A strain (LA29) of RSV (44) which induces cell proliferation and transformation at 37°C (46). The mutant pp60^{v-src} of LA29 is temperature sensitive for membrane association (3, 35) such that at 42°C, despite ongoing viral replication, cells proliferate but are not morphologically transformed (4, 45). We took advantage of this temperature sensitivity to examine both the proliferative and transforming properties of RSV LA29 throughout the 4 months which precede senescence of an infected chicken neuroretinal cell culture (LA29NR). We have analyzed the events leading up to senescence with regard to cellular morphology, mitotic index, and anchorage-independent growth, as well as expression of pp60^{v-src} and potential substrates of pp60^{v-src} tyrosine kinase activity. We have replicated the senescence phenomenon five times, with two or three replications of all assays described here.

LA29NR cells lose mitotic activity uniformly at 42 and 37°C after passage 12. LA29NR cells were prepared from embryonic day 7 chick neuroretinal tissue, infected at 37°C, and

found to express levels of neuron-specific enolase and glial fibrillary acidic protein, as evidence of their glial/neuronal phenotype (31). The majority of cells survived infection by continuing beyond the usual 2-week survival time for uninfected neuroretinal cells. LA29NR cells were maintained as monolayer cultures at the proliferative temperature of 42°C, fed every 2 to 3 days with Dulbecco's modified Eagle's medium-10% fetal calf serum, and subcultured weekly for approximately 4 months. Cultures maintained at 42°C were shifted to 37°C for 24 h immediately preceding all temperature comparison studies, because of rounding up and detachment of cells kept for more than a few days at the transforming temperature of 37°C. Mitotic index was determined by 24-h incorporation of 0.5 µCi of [³H]thymidine (Amersham) per ml, as previously described (31). As seen in Fig. 1, LA29NR cells remained greater than 90% mitotic at both 42 and 37°C at passage 12. However, between passages 13 and 15, the mitotic index dropped to 20% at both temperatures. Yet, the 80% population of nonmitotic cells remained adherent and intact. Cells could be maintained, as their numbers diminished, for 2 to 3 weeks at passage 15 until all cells died. Since mitotic changes were consistent at both proliferative and transforming temperatures, we sought to characterize several transformation parameters which might also be affected during the senescence phenomenon.

Reduced efficiency of anchorage-independent growth is an early sign of impending senescence. LA29NR cells possess the capability of anchorage-independent growth, characteristic of a transformed state (19), by forming large colonies in soft agar at 37°C. In order to assess anchorage-independent growth during the time period preceding senescence, we shifted the 42°C maintenance cultures to 37°C. Hard-agar base for each 60-mm dish consisted of Dulbecco's modified Eagle's medium with 0.63% Noble agar (Difco Laboratories, Detroit, Mich.) and 10% fetal calf serum (GIBCO). For the soft-agar overlay, 5 × 10³ cells per dish were suspended in 2 parts of hard agar to 1 part cloning medium (72% 1×, Dulbecco's modified Eagle's medium, 10% fetal calf serum, 12% tryptose phosphate broth, 4% chicken serum, 1× minimal essential medium vitamins, 8 µg of folic acid per ml, 50 µg of gentamicin per ml, 2.5 µg of amphotericin B). Cells were maintained at 37°C, with 2 ml of fresh soft agar being added every 5 days, and observed for a period of 4 to 5 weeks for colony formation. As seen in Fig. 2, there was a significant decrease in the size of soft-agar colonies after 2 weeks of growth, between passages 8 (Fig. 2A) and 12 (Fig. 2B), despite the 90% mitotic rate of LA29NR cells observed

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FIG. 1. Mitotic index of LA29NR cells. The mitotic index was determined by 24-h [^3H]thymidine incorporation and autoradiography. For each condition, three coverslips were scored for labelled nuclei. The mitotic index was calculated as follows: (number of labelled cells/total number of cells) $\times$ 100.

at passage 12. This would suggest a true decrease of anchorage independence, as opposed to solely a loss of cell viability. By passage 15, most cells remained as a single-cell suspension, with few colonies reaching the 4- to 5-cell stage shown (Fig. 2C).

Morphological changes precede senescence of LA29NR cells. Morphological changes characteristic of the transformed phenotype upon temperature shift from 42 to 37°C were documented throughout the in vitro life span of LA29NR cells (Fig. 3). The most visible of these morphological changes is an increase in the number of spheroidal cells shortly after a temperature shift to 37°C (1). Incubation for 24 h at 37°C caused a dramatic increase in the number of spheroidal cells at passage 4 (Fig. 3B), as well as at passage 13 (Fig. 3D). However, between passages 14 (Fig. 3F) and 15 (Fig. 3H), this morphological effect was virtually eliminated. In evaluating the number of spheroidal cells versus the number of nonspheroidal cells at 37°C, we found the ratios to be roughly 1:1 (spheroidal/nonspheroidal) at passages 4 and 13, 1:2 at passage 14, and 1:30 at passage 15. Also evident from Fig. 1 are some of the morphological changes which evolved at both temperatures, from passage 4 (Fig. 3A and B) to passage 15 (Fig. 3G and H). These changes included increased cell size and cell flattening. After 16 days in culture at 42°C, all passage 15 LA29NR cells developed unusual morphologies, which often included extensive process outgrowth, reminiscent of their mixed neuronal/glia cell origin, just prior to final senescence (Fig. 4).

Senescent LA29NR cells continue to express pp60^{v-src} but exhibit decreases in detectable phosphotyrosine residues of two specific proteins. The levels of pp60^{v-src} expression and tyrosine phosphorylation were examined by Western blot (immunoblot) analysis as described previously (15, 30, 31), with 25 μg of protein loaded per lane. Primary antibody to pp60^{v-src} (Oncogene Science, Inc.) at a 1:100 dilution or antiphosphotyrosine antibody PY20 (ICN Immunobiologicals) at a 1:1,000 dilution was utilized, followed by peroxidase-linked sheep anti-mouse immunoglobulin (Amersham) at a 1:300 dilution. Signals were generated onto autoradiographic film by ECL-enhanced chemiluminescence (Amersham) (43). As shown in Fig. 5A, after an initial lag of three or four passages, LA29NR cells expressed pp60^{v-src} at both

FIG. 2. Soft-agar colony formation. Soft-agar colonies were initiated at 37°C at passage 8 (A), 12 (B), or 15 (C) and photographed under light microscopy after 2 weeks of growth. Magnification, $\times 400$.

37 and 42°C. Although the amount of detectable pp60^{v-src} decreased somewhat by passage 15, the continued presence of pp60^{v-src} led to an examination of potential substrates of pp60^{v-src} tyrosine kinase activity. As seen in Fig. 5B, many phosphotyrosine residues were still present, even at late passage. However, two specific proteins with sizes of 36 and

FIG. 3. Temperature effects on transformed morphology of early- and late-passage LA29NR cells. LA29NR cells were grown at 42°C and then shifted to 37°C for 24 h to activate the temperature-sensitive RSV. (A) Passage 4, 42°C; (B) passage 4, 37°C; (C) passage 13, 42°C; (D) passage 13, 37°C; (E) passage 14, 42°C; (F) passage 14, 37°C; (G) passage 15, 42°C; (H) passage 15, 37°C. Original magnification, ×360.

69 kDa, respectively, exhibited the most dramatic decreases between passages 12 and 15 at both 42 and 37°C (Fig. 5C). Thus, one important potential pp60^{V-src} activity which seems to correlate with the onset of senescence involves either the underphosphorylation or the selective loss/degradation of two specific phosphoproteins. Since pp60^{V-src} tyrosine kinase activity has been implicated in RSV induction of chicken neuroretinal cell proliferation (25), underphosphorylation or functional loss of a phosphoprotein could be responsible for decreased proliferation as well as alterations of the transformed phenotype. For one purported substrate of pp60^{V-src} (29), a 36-kDa membrane-associated protein (28, 32), identified as the Ca²⁺ phospholipid-binding protein calpactin I (6, 9), there is precedent for the relationship between tyrosine phosphorylation and anchorage-indepen-

dent growth (20) as well as tumorigenicity (21). The heavy chain of calpactin I has been shown to be growth regulated in hamster fibroblasts (17). In addition, calpactin I protein has been detected in differentiating embryonic chicken lens (38) and could be a candidate for the 36-kDa phosphoprotein detected in our studies. Many such substrates of viral tyrosine kinases can be phosphorylated by endogenous cellular tyrosine kinases (16). However, the role of viral tyrosine phosphorylation has been demonstrated with regard to potential substrates (13),

FIG. 4. Morphologies of very-late-passage LA29NR cells. LA29NR cells were grown at 42°C and photographed under light microscopy 16 days after they were plated, at passage 15. (A and B) Representative morphologies which evolve immediately prior to cell death. Magnification, ×400.

FIG. 5. Altered expression of 69- and 36-kDa phosphoproteins is accompanied by continued expression of pp60^{V-src} during senescence. (A) Western blot analysis of LA29NR pp60^{V-src} expression as detected by pp60^{V-src} antibody, with a 30-s chemiluminescent exposure. (B) Western blot analysis of phosphotyrosine residues, as detected by antibody PY20 with a 4-min chemiluminescent overexposure to reveal the maximum number of proteins. Lane numbers refer to the passage number. p, permissive temperature (37°C); n, nonpermissive temperature (42°C). The arrow denotes a 69-kDa protein which migrated parallel with a 69-kDa marker; the arrowhead denotes the 36-kDa protein. (C) Magnified, isolated view of the 69-kDa (arrow) and 36-kDa (arrowhead) bands shown in panel B, with a 20-s chemiluminescent exposure.

such as the fibronectin receptor complex (14) or the phosphorylation of 95- and 135-kDa glycoproteins in RSV-transformed chicken embryo fibroblasts which correlated with transformed morphology and soft-agar growth (18). It is also significant that an inhibitor of tyrosine kinase activity, herbimycin A, can restore a nontransformed phenotype to v-src transformed cells (38) and that alterations in tyrosine phosphorylated proteins are implicated even in normal chicken neuroretinal cell development (33).

An interesting parallel exists in regressing RSV-induced chicken sarcomas (8), for which tumor regression has been attributed to a host T-cell-mediated immune response (41). Cultured regressing tumor cells demonstrate diminished pp60^{v-src} kinase activity (27), as well as a lack of transforming virions (42). Similarly, in the present study, chicken neuroretinal cells, transformed and maintained in vitro, display diminished viral effects with cell passage. In vivo, recruitment of new cells (12), as well as the continuing division of previously infected cells (36), has been shown to be important for growing tumors. Likewise, in vitro, the senescence phenomenon has led some investigators to continually replenish RSV-transformed chicken cell cultures with fresh, uninfected cells to maintain the infected cell populations (26). Thus, loss of RSV effects on chicken cells appears to be a common motif, both in vivo and in vitro.

It is clear from our studies that many viral effects decrease sharply between passages 12 and 15, rather than attenuating gradually over the entire life span of LA29NR. These changes include a decrease in mitotic activity at both permissive and nonpermissive temperatures, a loss of transformed phenotypic characteristics, and finally, the appearance of unusual morphologies just prior to cell degradation and death. For the chicken neuroretinal cell system, we can now characterize senescent cells with regard to specific neuroretinal phenotypes.

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