Modulation by Normal Serum Factors of Kirsten Murine Sarcoma Virus-induced Transformation in Adult Rat Cells Infected in Early Passage

Nelly Auersperg,1 Craig H. Siemens, Gerald Krystal, and Sigrid E. Myrdal2

Department of Anatomy, The University of British Columbia, Vancouver, British Columbia V6T 1W5 [N. A., C. H. S., S. E. M.], and Terry Fox Laboratory, B. C. Cancer Research Centre, Vancouver, British Columbia V5Z 1L3 [G. K.], Canada

ABSTRACT

Adult rat adrenal cells, infected with Kirsten murine sarcoma virus in early passage, transform consistently in 10% fetal bovine serum (FBS)-supplemented medium. Substitution of 3% horse serum (HS) for FBS reverses early foci and delays transformation. The influence of the serum on DNA synthesis, anchorage dependence, tumorigenicity, and subcellular M, 21,000 transforming protein (p21) distribution was followed from infection in passage 1 to complete transformation. In FBS, increased expression of p21 preceded other evidence of transformation. Subsequently, p21-positive cells transformed morphologically, but initially their growth paralleled that of coexisting untransformed cells. Foci formed at passages 5 to 10, and the cells became anchorage independent and tumorigenic at passages 10 to 20. As transformation in FBS progressed, p21 relocated from a diffuse distribution to sites of retraction from substrata and then to ruffles and lamellae on cellular processes. Early in transformation, HS-medium reduced proliferation of morphologically normal and morphologically transformed p21-positive cells. This effect was counteracted by the addition of FBS or the M, 50,000 to 100,000 fraction of FBS. Fully transformed, tumorigenic cells grew rapidly in both sera but, if transferred from FBS to HS, became more anchorage and density dependent, and p21 relocated from cell processes to the cell bodies. In immortal lines, the substitution of HS for FBS accelerated rather than delayed the progression of Kirsten murine sarcoma virus-induced transformation. These results show that Kirsten murine sarcoma virus-induced transformation of adult presenescent cells is controlled by physiological factors to which immortal cells appear refractory. The changes in subcellular distribution of p21 during transformation parallel the expression of some, but not other, transformation parameters and suggest a possible association of p21 with increased membrane activity.

INTRODUCTION

Much of the information that has accumulated about the role of retroviral oncogenes in carcinogenesis is based on model systems which use cultured immortal lines or embryonic cells as the targets. However, while the basic mode of action of any one oncogene may be similar in all cells, its effects on phenotypic expression ultimately depend on the differentiation and the physiological state of the target cells. Therefore, the relevance of oncogenes in clinical cancer can be investigated more directly in cultures of adult tissues in early passage (1–5). Such presenescent cells are distinguished from immortal lines by a greater competence to express tissue-specific properties in response to the appropriate growth factors, and by an exquisite sensitivity to environmental regulatory mechanisms. They are also more representative of target cells in carcinogenesis in the intact adult organism than are embryonic cells, because they lack many of the features that embryonic and malignant cells have in common. Such features may predispose the embryonic cells to transformation and may mask some of the steps in carcino-

5715
MODULATION OF v-Ki-roj-INDUCED TRANSFORMATION

Inc., New York, NY) at 37°C in 5% CO₂/air. Outgrowths were dissociated with trypsin (0.125% in Ca-, Mg-free Hanks' BSS) and subsequently grown in medium supplemented with 10 to 25% FBS (FBS-medium). KiMSV was harvested from supernatant medium of the KiMSV-transformed cell line NRK 58967 and added to the adrenal cultures in the first passage. At various points in the subsequent transformation process, parallel sublines were established and maintained thereafter in medium supplemented with either 3% HS or with 10% FBS. All observations made in the present study were tested on replicate cultures of at least 2 of the 4 independently established lines, which were identified as lines TRA 13/V1(1), TRA 13/V1(2), TRA 16/III, and TRA 12/VI. Immortal lines used for comparison with the early passage adrenal cultures included NRK cells (passages 4 to 52) and NRA-E cells, an immortal line of untransformed rat adrenal cells (passages 15 to 19). The nonneomurigenic rat adrenal line NREA-E was derived from surviving cells of an adrenal culture undergoing senescence after approximately 6 weekly passages. Compared to presenescent adrenal cells (9), NREA-E cells were more monolayered and contact inhibited, and they did not secrete detectable extracellular matrix. NREA-E cells were not tumorigenic for 1 yr after inoculation into rats, under conditions where tumors arose in 100% of animals within 2 wk when inoculated with KiMSV-transformed cells (10). Subconfluent cultures of the immortal lines were infected with KiMSV and processed as described above for early passage adrenal cultures.

Morphology and Growth Pattern. Transformation in culture was defined by alterations in shape, focal piling up or retraction of cells, and sudden increases in growth rate and pH reduction. The cultures were scored as completely transformed when no areas with normal appearing cells could be identified. Criteria used for reversion from a transformed to an untransformed phenotype were increased cell flattening, monolayering, and complete or near-complete cessation of cell proliferation.

Serum Fractionation. FBS (3- and 20-ml aliquots) was fractionated at 4°C by size on 135-ml and 610-ml bed volume Sephadex G150 columns, respectively, and equilibrated with PBS, and 6 equal sized pools (20 and 100 ml each, respectively) were dialyzed against Waymouth's medium. Fifty % of this dialysate was added to 50% of Waymouth's medium/6% HS for a final concentration of 50% fraction/3% HS/Waymouth's medium. The fraction in the final medium was equivalent to the material present in approximately 8% FBS.

Preliminary experiments showed that there was no activity in the void volume peak, which was included in Fraction 1. Cells of lines TRA 13/V1(1) and TRA 13/V1(2) were maintained in triplicate 2-cm² wells on HS-medium, FBS-medium, dialyzed FBS-medium, and media with each of the 6 fractions from passage 2 to complete transformation. All cultures were plated in HS-medium and transferred to the test media at 2-wk intervals after infection until they were fully transformed, and each set was maintained for 4 wk. The only consistently active fraction was in the molecular weight range of albumin (M₅, 50,000 to 100,000).

Therefore, this material was fractionated further on a 5.0- x 1.0-cm Affi-Gel Blue column (Bio-Rad), equilibrated with PBS, into albumin-void volume peak, which was included in Fraction 1. Cells of lines TRA 13/V1(1), TRA 16/III, and TRA 12/VI in passages 2 to 5 and 25 to 27. The animals were kept for 2 mo or until they formed easily palpable, progressively growing tumors.

Immunoaffluorescence. Sparse monolayers of lines TRA 12/VI in passages 4 to 6, TRA 16/III in passage 3, and TRA 13/V1(1) in passages 5 to 7 and 29 and 30 were plated onto glass coverslips in HS-medium, and 24 h later, they were changed to the appropriate culture medium. Two to 5 days later, the coverslips were transferred directly from culture medium to 3.7% formaldehyde in pH 7.4, 0.02 M PBS, 2 min, washed twice in PBS, left 20 min or longer in −20°C absolute methanol, brought to room temperature in absolute acetone over 30 min, and air dried. The specimens were rehydrated in PBS and then stained with rat monoclonal antibody Y13-259 (an antibody directed against p21, the transforming protein encoded by KiMSV (12), generously donated by Dr. M. Fuhr, followed by fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (whole molecule), IgG fraction (Miles Laboratories, Inc., Elkhart, IN) as previously described (13).

RESULTS

As in previous experiments (6), uninfected and recently infected adrenal cells modulated morphologically and in growth rate in response to changes in serum supplements; in FBS-medium, the cells resembled fibroblasts and grew rapidly, while in HS-medium, they were epithelial and near stationary. Early in the transformation process, there first appeared a subpopulation of morphologically transformed cells. In FBS-medium, these cells initially did not form foci but, rather, their growth rate and growth pattern paralleled the growth of the coexisting normal cells in the population. Foci appeared in FBS-medium after 5 to 10 weekly passages. In HS-medium, the cultures remained stationary or near stationary for several weeks, and focus formation was delayed compared to FBS-grown cultures. Transfer from FBS-medium to HS-medium shortly after infection delayed the appearance of foci. In cultures with early foci, HS-medium caused reversion to a morphologically normal, slow growing phenotype. Fully transformed cells retained a transformed morphology and grew rapidly in both sera (Fig. 1; Refs. 6 and 14).

To examine the effect of immortalization on the responsiveness of cells to the serum supplements, subconfluent cultures of NRK and NREA-E cells were infected with KiMSV. Uninfected NRK cells and NREA-E cells were near stationary in HS-medium, like early passage cultures. However, the inhibitory effect of HS-medium on focus formation by early passage cells was absent in the immortal lines. Foci appeared in NRK and NREA-E cultures on both sera by 4 to 7 days postinfection. The numbers of foci in HS-medium exceeded those in FBS-medium from the beginning in the NREA-E cultures and by Days 10 to 11 in NRK cultures (Table 1). Thus, HS-medium enhanced rather than delayed focus formation by immortal cells as compared to focus formation in FBS-medium. Also in contrast to early passage cultures, HS-medium did not cause the reversion of early foci formed in FBS-medium (data not shown).

Anchorage and Density Dependence. Anchorage independence was assayed by agarose technique of MacPherson and Montagnier (11). Assays were carried out over 14-day periods, at 10⁴ to 3.3 x 10⁴ cells per 60-mm dish in lines TRA 13/V1(1) at passages 4 to 7 and 25 to 36, TRA 16/III at passages 3 to 6, and TRA 12/VI at passages 19 and 20. The cultures were maintained on either FBS-medium or HS-medium for at least 2 wk prior to each assay. Unless stated otherwise, the agarose was supplemented by the same type of serum as the liquid medium in which the cells had grown. In 2 sets of experiments, duplicate dishes of TRA 12/VI cells were seeded on plastic at densities of 5 x 10⁴, 5 x 10⁵, and 3.3 x 10⁵ cells per 60-mm culture dish, in both media. Anchorage independence assays (3.3 x 10⁵ cells per dish) were run in parallel. Colonies were counted after 14 days.

Tumorigenicity. Four to 6-wk-old Fischer 344 rats were irradiated with 400 R or 36 h later were given injections s.c. of approximately 1 to 2 x 10⁵ cells of lines TRA 13/V1(1), TRA 16/III, and TRA 12/VI in passages 2 to 5 and 25 to 27. The animals were kept for 2 mo or until they formed easily palpable, progressively growing tumors.
Fig. 1. Cultures of rat adrenal cells [line TRA 13/VI(1)] infected with KiMSV in passage 1. a, c, and e, FBS-medium; b, d, and f, HS-medium. a and b (2-wk culture/3rd passage), rapid growth of fibroblast-like cells in FBS and stationary epithelial-like cells in HS. No foci, though p21-positive cells can be demonstrated by immunofluorescence at this stage. c (6-wk culture/7th passage) and d (10-wk culture/4th passage), beginning of focus formation in both media (arrowheads). e and f (20-wk culture/22nd passage), fully transformed cells. Note reduced adhesion to plastic and increased intercellular aggregation in HS and numerous cellular processes in FBS. Phase microscopy, × 200.

Table 1 Effect of serum supplements on focus formation following KiMSV infection in cultures of adult rat adrenal cells in early passage (TRA 7/X-1) and after immortalization [NRK, NRA-E(1), and NRA-E(2)]

NRA-E(1) and (2) are separately infected lines derived from line NRA-E. All early passage cultures were infected with the highest available virus concentration. Virus concentrations for infection of immortal lines were chosen so as to give focus counts in FBS-medium similar to those in early passage cultures. All cultures were infected when near confluent while on FBS-medium; half of them were transferred to HS-medium 1 to 2 days later, while the others were refed with FBS-medium at the same time. Foci were counted 8 wk (TRA-7/X-1), 11 days (NRK), 7 days [NRA-E(1)], and 4 days [NRA-E(2)] after infection.

<table>
<thead>
<tr>
<th>Virus concentration (FFU/culture)</th>
<th>TRA 7/X-1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NRK</th>
<th>NRA-E(1)</th>
<th>NRA-E(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS-medium</td>
<td>HS-medium</td>
<td>FBS-medium</td>
<td>HS-medium</td>
<td>FBS-medium</td>
</tr>
<tr>
<td>4.2 × 10⁴</td>
<td>130, 123</td>
<td>0, 0</td>
<td>---&lt;sup&gt;c&lt;/sup&gt;</td>
<td>---</td>
</tr>
<tr>
<td>2.6 × 10⁴</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2.6 × 10³</td>
<td>---</td>
<td>---</td>
<td>26, 28</td>
<td>76, 65</td>
</tr>
<tr>
<td>2.6 × 10²</td>
<td>---</td>
<td>---</td>
<td>448, 470, 1216, 1432</td>
<td>4, 2</td>
</tr>
</tbody>
</table>

<sup>a</sup> FFU, focus-forming units as assayed in NRK cultures on 5% calf serum; TNTC, too numerous to count.

<sup>b</sup> For origin of line TRA 7/X-1, see Ref. 10.

<sup>c</sup> ---, not done.
Table 2 Incorporation of [3H]thymidine into uninfected and KiMSV-infected adult rat adrenal cells [line TRA 13/VI(1)] (5 x 10^6 cells/microtiter well; 4-h incubations with 1.0 μCi of [3H]thymidine per 0.1 ml of culture medium per well)

The cells were plated in HS-medium. Twenty-four h later, medium was changed in all wells to either HS-medium or FBS-medium. After 20 h, [3H]thymidine was added for 4 h, and then the experiments were terminated. Background counts (cell-free wells with medium and [3H]thymidine) were below 100 dpm for all groups.

<table>
<thead>
<tr>
<th>Wk/passage</th>
<th>In culture</th>
<th>After infection</th>
<th>Phenotype (FBS)</th>
<th>dpm/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FBS-medium</td>
<td>HS-medium</td>
</tr>
<tr>
<td>3/4</td>
<td>Uninfected</td>
<td>Normal</td>
<td>6,387 ± 505^a</td>
<td>486 ± 109</td>
</tr>
<tr>
<td>4/4</td>
<td>Uninfected</td>
<td>Normal</td>
<td>5,602 ± 403</td>
<td>1,879 ± 684</td>
</tr>
<tr>
<td>4/4</td>
<td>2/3</td>
<td>Partially transformed, growing</td>
<td>4,938 ± 462</td>
<td>1,088 ± 185</td>
</tr>
<tr>
<td>6/6</td>
<td>4/5</td>
<td>Partially transformed, senescent</td>
<td>192 ± 63</td>
<td>16 ± 12</td>
</tr>
<tr>
<td>7/7</td>
<td>5/6</td>
<td>Partially transformed, senescent</td>
<td>780 ± 86</td>
<td>62 ± 19</td>
</tr>
<tr>
<td>22/27</td>
<td>20/26</td>
<td>Fully transformed</td>
<td>18,374 ± 5,444</td>
<td>18,649 ± 1,489</td>
</tr>
</tbody>
</table>

^a Mean ± SD.

Table 3 Tumorigenicity and anchorage independence of Kirsten-MSV-infected adult rat adrenal cells [line TRA 13/VI(1)]

For anchorage independence, duplicate 60-mm dishes were assayed for each of the 5 variables tested, on 2 to 4 separate occasions, and the results were pooled. One of several cell concentrations tested is shown.

<table>
<thead>
<tr>
<th>Wk after infection</th>
<th>Morphology (FBS)</th>
<th>No. of rats tumorigenic (1-2 x 10^6 cells/rat)/total</th>
<th>Anchorage independence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FBS</td>
<td>HS</td>
</tr>
<tr>
<td>1</td>
<td>Normal</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-3</td>
<td>Few foci</td>
<td>4/4 (23)^f</td>
<td>0</td>
</tr>
<tr>
<td>4-5</td>
<td>Foci</td>
<td>2/5 (14-21)</td>
<td>0</td>
</tr>
<tr>
<td>18+</td>
<td>Transformed</td>
<td>4/4 (&lt;7)</td>
<td>4/4 (&lt;7)</td>
</tr>
</tbody>
</table>

^a Seeded as small aggregates rather than as single cells.
^b HS-grown cells seeded in FBS-supplemented agarose.
^c FBS-grown cells seeded in HS-supplemented agarose.
^d --, not done.
^e Regressed.
^f Numbers in parentheses, latent periods (days).
^g Mean ± SD of the number of colonies/dish (3.3 x 10^6 cells/dish).

Table 4 Effect of FBS, HS, and FBS-fractions on the onset of rapid replication, morphological transformation, focus formation, and DNA synthesis during progression of transformation

The onset of rapid replication, morphological transformation, and focus formation is given in weeks from addition of test serum.

<table>
<thead>
<tr>
<th>Serum + fraction no.</th>
<th>Molecular wt of fraction × 10^-3</th>
<th>Molecular wt of fraction</th>
<th>RR</th>
<th>MT</th>
<th>FF</th>
<th>% of DNA synthesis in FBS-medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FBS</td>
<td></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>100 ± 26^c</td>
</tr>
<tr>
<td>3% HS</td>
<td></td>
<td>12</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>3% HS + 1</td>
<td>100-150</td>
<td>12</td>
<td>8</td>
<td>12</td>
<td>8</td>
<td>100 ± 26^c</td>
</tr>
<tr>
<td>+2</td>
<td>50-100</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>100 ± 26^c</td>
</tr>
<tr>
<td>+3</td>
<td>25-50</td>
<td>11</td>
<td>8</td>
<td>11</td>
<td>8</td>
<td>100 ± 26^c</td>
</tr>
<tr>
<td>+4</td>
<td>10-25</td>
<td>12</td>
<td>9</td>
<td>12</td>
<td>9</td>
<td>100 ± 26^c</td>
</tr>
<tr>
<td>+5</td>
<td>1-10</td>
<td>11</td>
<td>9</td>
<td>11</td>
<td>9</td>
<td>100 ± 26^c</td>
</tr>
<tr>
<td>+6</td>
<td>&lt;1</td>
<td>12</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td>100 ± 26^c</td>
</tr>
</tbody>
</table>

^c Line 13/VI(2). KiMSV infected at 2 wk, transferred from FBS to HS at 2 wk, fractions added at 4 wk.
^d Incorporation of [3H]thymidine over 4 h after 6 wk in culture, expressed as percentage of FBS-grown cells.

DNA Synthesis. Within 24 h after transfer to different serum supplements, DNA synthesis in HS-medium was significantly reduced as compared to cells in FBS-medium in both uninfected cultures and in incompletely transformed cultures of both lines tested (Table 2). The difference was greatest at 6 to 7 wk, when parallel uninfected cultures were undergoing senescence. In fully transformed cultures, thymidine uptake was similar in both sera.

Tumorigenicity, Anchorage Independence, and Density Dependence. In cultures at the stage of focus formation, cells were tumorigenic after latent periods of 14 to 21 days (Table 3). Fully transformed cells were highly tumorigenic, with latent periods of less than 7 days, regardless of the serum that they had been cultured in. In FBS-medium, cells from incompletely transformed, tumorigenic cultures formed no colonies in agarose, although many were morphologically transformed and p21 positive. Anchorage-independent cells were detected only when the cultures were completely transformed. Interestingly, cultures maintained in HS-medium for 2 wk or longer formed few or no colonies in HS-supplemented agarose even when fully transformed by the criteria of tumorigenicity, morphology, and rapid growth in HS-medium under standard conditions. This lack of growth in HS-agarose was not due to inadequate nutritional conditions, because the cells grew if they were seeded in HS-agarose as small aggregates rather than singly. More colonies formed if cells maintained on HS-medium were transferred to FBS-agarose, and when cells maintained on FBS-medium were transferred directly to HS-agarose. However, under both conditions, anchorage-independent cells were not detected. Interestingly, cultures maintained in FBS-medium for 2 wk or longer formed few or no colonies in FBS-supplemented agarose even when fully transformed by the criteria of tumorigenicity, morphology, and rapid growth in FBS-medium under standard conditions. This lack of growth in FBS-agarose was not due to inadequate nutritional conditions, because the cells grew if they were seeded in FBS-agarose as small aggregates rather than singly. More colonies formed if cells maintained on FBS-medium were transferred to FBS-agarose, and when cells maintained on FBS-medium were transferred directly to HS-agarose. However, under both conditions, anchorage-independent cells were not detected.
Fig. 2. p21 distribution in adrenal cells [line TRA 13/V(1)] infected with KiMSV in passage 1, fixed 5 wk later. a, d, and g, phase microscopy; b, c, e, f, h, and i, indirect immunofluorescence with rat anti-rat v-Ki-p21 monoclonal antibody followed by fluorescein isothiocyanate-conjugated rabbit anti-rat IgG. a, b, and c, uninfected control culture. d, e, and f, FBS-medium. g, h, and i, HS-medium. Fluorescence in c, f, and i is enhanced over that in b, e, and h, respectively, by reducing printing time. In FBS-medium, p21 is amplified and diffusely distributed in morphologically normal cells (compare X in c and f), but it is especially prominent at the peripheral adhesive site of a morphologically transformed cell (e and f). where it is distributed in a complex, heterogeneous manner. In HS-medium, p21 is also amplified in morphologically normal cells (i, X). In a morphologically transformed cell, the bulk of fluorescence is associated with the main cell body rather than with adhesive footpads (h and i, A). Note increased fluorescence at sites of retraction from plastic (i, B). × 400.
of these conditions, the number of colonies was significantly reduced compared to FBS-grown cells plated in FBS-agarose (Table 3). Fully transformed FBS-grown TRA 12/VI cells were moderately density dependent when grown on plastic. At a seeding density of $5 \times 10^3$ cells per 60-mm dish, their cloning efficiency was 10 to 20%; at seeding densities of $5 \times 10^3$ and $3.3 \times 10^4$ cells per dish, colonies were too numerous to count. In contrast, HS-grown cells formed no colonies at seeding densities of $5 \times 10^3$ and $5 \times 10^4$ cells per dish. At $3.3 \times 10^4$ cells per dish, the cloning efficiency was less than 1%; however, by 2 wk there were many proliferating cells though colonies were too indistinct to be counted. Agarose cultures, grown in parallel, differed between FBS-medium and HS-medium as shown in Table 3 for line TRA 13/VII(1).

Effects of FBS Fractions. In 2 independent culture series, only Fraction 2, containing the $M$, 50,000 to 100,000 proteins, consistently promoted growth to a level intermediate between HS-medium and FBS-medium and accelerated the appearance of morphologically transformed cells and of foci (Table 4). The additional activity in Fraction 1 in Experiment 2 may have been the result of an overlap in pools. The effects of the albumin-free and albumin-containing subfractions of Fraction 2 were intermediate between those of FBS- and HS-medium. In 2 sets of experiments, levels of DNA synthesis were 37 and 40% of the level in FBS-medium with complete Fraction 2, 8 and 23% with the albumin-containing subfraction, 7 and 17% with the albumin-free subfraction, and 5 and 13% in HS-medium alone. Standard deviations were $<15\%$ of the mean in all groups. Pure albumin, equivalent to the amount in FBS-medium, reduced DNA synthesis to 0 to 0.5% of DNA synthesis in FBS-medium.

Subcellular Distribution of p21. Within 2 wk after KiMSV infection, p21 was amplified and diffusely distributed in some flat, morphologically untransformed cells (Figs. 2 to 4). In cells undergoing morphological transformation, it was often concentrated in regions of cellular retraction. In addition, there was a small subpopulation of brightly fluorescent, morphologically transformed cells present in early passage cultures in both sera. In these cells, most of the fluorescence appeared to be subplasmalemmal, in the region of the main cell body, and in FBS-medium, fluorescence also appeared in cellular extensions. Five wk after infection, the proportion of morphologically transformed, brightly fluorescent cells was increased in both sera. In addition, the proportion of morphologically normal cells with increased fluorescence had increased. This was particularly evident in cultures on HS-medium, suggesting that morphological reversion in HS-medium was not associated with a reduced expression of p21. The intensity of fluorescence varied widely among morphologically untransformed cells, suggesting intracellular heterogeneity in the amounts of p21 expressed. As transformation progressed, most of the fluorescence remained in the main cell bodies of HS-grown cells; in FBS-medium, however, p21 became increasingly concentrated in peripheral cellular extensions, particularly at sites of adhesion to plastic and to other cells. At such sites it was distributed in a heterogeneous, complex pattern, which frequently colocalized with ruffles, blebs, and lamellae seen by phase microscopy.

In fully transformed, tumorigenic cultures, over 90% of cells were p21 positive in both sera. However, the subcellular distribution of p21 remained different depending on the serum supplement, as described above (Fig. 4).

DISCUSSION

It has become apparent that the effects of retroviral oncogenes can be suppressed in the continued presence of elevated levels of presumably unaltered transforming proteins (15, 16). Suppression can be achieved by exogenous agents (17), differentiation (18), serum components (7), and the embryonic environment in vivo (19). Our study demonstrates that physiologically occurring environmental factors regulate the progression of v-Ki-ras-induced transformation in populations of freshly explanted adult presenescent cells, in the continued presence of elevated p21 levels. The developmental and physiological state of these cells is analogous, in many respects, to the state of target cells acted on by carcinogens in the intact organism.

When presenescent cells were maintained in FBS-medium, amplified p21 and morphological changes appeared within one passage after infection but, in contrast to immortal lines (20), further progression was delayed. This limited transforming potential of the viral Ki-ras oncogene resembles the transforming potential of c-ras in primary embry cultures (21). In the present study, the onset of uncontrolled growth by the transforming cells may have related to their immortalization (21) and to the senescence of coexisting untransformed cells which occurs after approximately 6 passages. Other evidence suggests that the expression of the neoplastic phenotype can be controlled by interactions with normal cells and their products (19, 22–25). The acquisition of anchorage and serum independence frequently reflects altered GF requirements and the onset of autocrine GF production (26–28). In FBS-grown cells, the acquisition of anchorage and serum independence coincided with increased ruffling, which has recently been shown to be a sensitive indicator of GF activity (29).
MODULATION OF v-Ki-ras-INDUCED TRANSFORMATION

In early passage cultures, HS-medium delayed the progression to fully transformed cultures. It is unlikely that this delay related to a difference in the spread of KiMSV. In the type of system used here, the excess helper virus present would be expected to block secondary infection by KiMSV after the initial exposure. A simple explanation for the effect of HS-medium would be that it was nutritionally inferior to FBS-medium. However, although HS-medium also slowed the growth of uninfected immortal cells, it enhanced rather than inhibited focus formation in immortal lines newly infected with KiMSV. It appears therefore that the immortal cells had undergone predisposing changes prior to infection (2) which, in conjunction with v-ras oncogene expression, rendered the cells independent of the growth inhibition by HS-medium. Others have shown that stringent culture conditions inhibit the transformation efficiency of presenescent cells more than of immortal lines (30). Since in presenescent adrenal cultures HS-medium prevented replication and focus formation by morphologically transformed p21-positive cells, this block to malignant progression must have been distal to p21 synthesis. The preliminary FBS fractionation results reported here suggest that an FBS component in the M, 50,000 to 100,000 range, which is not albumin but may be albumin bound, can overcome some of the inhibitory influence of HS-medium.

Although the growth rate and tumorigenicity became independent of serum supplements in fully transformed cells, other aspects of the transformed phenotype did not. Transfer of fully transformed cells between FBS-medium and HS-medium caused translocations of the bulk of p21 between peripheral ruffles and the main cell bodies, respectively. Thus, FBS-grown cells exhibited an intracellular p21 distribution characteristic of the fully transformed phenotype (13), while HS-grown cells did not. In addition, HS-grown cells were considerably more density dependent and formed few colonies in agarose compared to FBS-grown cells. The reduced ability of HS-grown cells to form colonies in HS-agarose likely reflected their inability to clone in this culture medium. However, in addition, their reduced capacity to form colonies in FBS-supplemented

Fig. 4. p21 distribution in fully transformed, tumorigenic adrenal cells [line TRA 13/V(1)] 22 wk after infection. a, b, and c, phase microscopy; d, e, and f, immunofluorescence as in Fig. 2. a and d, uninfected control cells; b and e, FBS-medium; c and f, HS-medium. FBS-grown cells have prominent processes with ruffled adhesive footpads, where most of the fluorescence is localized. In HS-medium, the cells retract and adhere more to one another, and p21 appears associated mainly with the cell bodies. × 400.
agarose suggests that maintenance in HS-medium rendered the cells more anchorage dependent. All of these phenotypic modulations were reversible, and in their kinetics resembled the inheritance of acquired characteristics described by Rubin et al. (31). The serum-induced difference in density and anchorage dependence suggests that even fully transformed adrenal cells may produce insufficient growth factors for complete autonomy from sera, and that such factors may be supplied by FBS. During transformation in FBS, the cells formed tumors before colony formation in agar was demonstrable. The late onset of colony formation in agarose by the transforming cells may have reflected the development of tumorigenicity prior to anchorage independence; alternatively, since considerably more cells were injected for tumorigenicity testing than were used for agar cloning, the early tumors may have arisen from a small subpopulation of anchorage-independent tumorigenic cells, which were not detected in the agarose assays. In either case, the results of this study indicate the limitations of growth in agarose as an indicator of tumorigenicity.

The subplasmalemmal location of p21 has been reported previously (32–34). In our model system, increased viral p21 levels alone were insufficient to induce morphological transformation (7), though they are sufficient to induce this change in some immortal cells (35–37). The codistribution of p21 with areas of rounding, with footpad-associated ruffles, and with blebs and lamellae suggests an association of p21-rich subplasmalemmal regions with changing cell shapes and with increased membrane activity. The complex, heterogeneous intracellular distribution of p21 and the changes which this distribution underwent with progression and in response to sera should provide clues about associated changes in the cytoskeleton (38) and in membrane function.

Our results illustrate the dependence of the viral Ki-ras oncogene on components of FBS to efficiently initiate and complete transformation of presenescent adult cells in culture. It is possible that, in this transformation process, senescence of incompletely transformed cells is delayed (39) as a result of v-ras oncogene-induced production of GFs. However, the role of the v-ras oncogene here appears to be more than one of delaying senescence, since in our experience immortal lines derived from rat adrenal cells do not transform spontaneously.

Our observations are in keeping with other studies, based on biochemical analysis of whole cell populations, that have shown p21 levels to be increased early in the transformation process (7). Of all known oncogenes, ras has been most frequently found activated in human malignancies. In addition, p21 is amplified in premalignant clinical lesions (40, 41). The mechanisms which control the progress of such early lesions to fully malignant neoplasms are unknown. It would appear possible that they are related to the mechanisms which control the transformation sequence of KiMSV-infected, presenescent adult cells in culture.

ACKNOWLEDGMENTS

We thank Judith Black for excellent technical assistance.

REFERENCES

MODULATION OF v-Ki ras-INDUCED TRANSFORMATION


Modulation by Normal Serum Factors of Kirsten Murine Sarcoma Virus-induced Transformation in Adult Rat Cells Infected in Early Passage

Nelly Auersperg, Craig H. Siemens, Gerald Krystal, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/11/5715

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.