CELL TRANSFORMING GENES AND TUMOR PROGRESSION:  
IN VIVO UNIFIED SECONDARY PHENOTYPIC CELL CHANGES

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We have earlier shown that Syrian hamster cells spontaneously transformed in vitro during in vivo progression, acquire in 1 step, along with highly increased tumorigenicity, 2 new properties characterizing the [H2O2CA + PGE5] phenotype, i.e., a high H2O2 catabolizing (antioxidant) activity and the ability to release PGE2 upon contact with NK cells. In contrast, RSV-SR-(v-src)-transformed cells acquire the [H2O2CA + PGE5] phenotype and high tumorigenicity during in vitro transformation, i.e., without preliminary in vivo selection. In the present study, the possible influence of different transforming genes on the rates of subsequent in vivo tumor progression was studied using cells in vitro transformed by SV40, BAV-3, or transduced by activated genes Ha-ras, p53, myc and bcl-2. The expression of the [H2O2CA + PGE5] phenotype, the extent of tumorigenic and spontaneous metastasizing activities were examined before and during in vivo cells selection in s.c. growing tumors. Our results demonstrate that: (1) after in vitro transformation all cell lines (except v-src) were negative for the expression of [H2O2CA + PGE5] phenotype and remained equally low-tumorigenic; (2) independently of the types of genes initially transforming the cells, in vivo tumor progression was consistently leading to the replacement of parental cells by cells expressing the [H2O2CA + PGE5] phenotype, to 30–200 times increased tumorigenicity and less frequently to metastasizing; (3) the time necessary for selection of cells expressing this phenotype was the same (about 180 days in vivo) for all transformants, except bcl-2; the latter reaching similar values after a significant delay. Thus, common secondary src-like phenotypic cell changes, regardless of initially cell transforming genes are necessarily selected during tumor progression in vivo. Int. J. Cancer 75:277–283, 1998.

The role of transforming genes in subsequent tumor progression may be studied using 2 main approaches. The first is the determination of the expression of different oncogenes in the tumors of different origin and at the different stages of progression (i.e., tumors in situ, early growing and advanced tumors and their metastasis). This approach is widely used in the study of spontaneous human tumors and it permits the correlation, to some extent, of the clinical and patho-morphological stages of tumor development with the activity of particular oncogenes. However, it provides scarce information on the role of oncogenes which originally transformed these cells on tumor progression as well as on their role on the in vivo selection of tumor cells. The second is the comparative study of individual cell lines transformed in vitro by oncogenes and their descendant cell variants selected in vivo. This approach permits to follow the dynamics of tumor progression and the changes regularly occurring in the malignant behaviour of individual tumors, i.e., the extent of tumorigenic and spontaneous metastasizing activities (TGA, SMA), as well as of different secondary cell changes, which might be selected during the in vivo growth of the tumors.

This latter approach was used in the present study; it is based on our previous observations on the in vivo progression of 2 types of tumors, originating from in vitro spontaneously- and RSV-SR-transformed hamster embryo cells, our main finding being that in vivo selection of highly-tumorigenic variants of spontaneously transformed cells coincides with the acquisition of an in vitro detectable new and stable cell phenotype, designated [H2O2CA + PGE5]. This phenotype represents the co-expression of 2 biochemically different cell properties: (1) highly increased antioxidant activity of cells, determined by their increased rate of H2O2-catabolizing activity (H2O2CA), as well as by increased resistance to damage by H2O2 (H2O2V); (2) the acquired ability to immediately release PGE2 (PGE5) upon contact interactions with NK cells. It was surprising that these 2 new properties in all variants of highly tumorigenic spontaneously transformed cells were acquired in vivo in one step: no intermediate variants were noticed. Expression of these properties coincided with significantly increased resistance of the cells to cytotoxic actions of macrophages and of NK cells. Even more surprising was that in one step and without any selection in vivo, the same phenotype (together with extremely high TGA and even SMA) was expressed by the cells transformed in vitro by RSV-SR (v-src) (Deichman and Kashleva, 1987; Deichman et al., 1989a,b, 1990; Kluchareva et al., 1992).

In connection with these data, it was of interest to examine the rates of progression in vivo and the characteristics of tumors originating from the cells transformed in vitro by different oncogenes. Therefore, our present study was focused on the following 3 topics: (1) the possible influence of different transforming genes on the original levels of TGA and SMA of in vitro transformed cells and on the possible expression of the [H2O2CA + PGE5] phenotype; (2) the dynamics of the in vivo progression of tumors growing s.c., originating from cells spontaneously transformed in vitro, or by oncoviruses (RSV-SR; SV40; BAV-3), or transduced by different activated genes (Ha-ras; myc; p53[375] and bcl-2); (3) identification of the possible secondary phenotypic cell changes (including the [H2O2CA + PGE5] phenotype) acquired by these cells in s.c. growing tumors during in vivo progression.

Our data show that independently from the type of transforming genes (LTS40, E1a and E1b, Ha-ras, p53[375], myc and bcl-2), all in vitro transformed cells remained equally low-tumorigenic and negative in the expression of the [H2O2CA + PGE5] phenotype, while their in vivo selected malignant descendant cell variants necessarily express a new [H2O2CA + PGE5] phenotype and highly increased TGA. The acquisition of these properties allows a clear distinction of tumor cells from the parental in vitro transformed ones and strikingly reminiscent of the in vitro v-src-related cell phenotype. The expression of [H2O2CA + PGE5] phenotype pre-

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cedes or coincides with a 30–200 times increased tumorigenic activity of the selected cell variants and it is expressed in the cells that metastasize spontaneously.

MATERIAL AND METHODS

Cells

Syrian hamster embryo cells spontaneously transformed in vitro (STHE strain), or by oncogenic viruses: SV40 (HE-WTSV40 strain), BAV-3 (HE-4 strain) and RSV-SR (HET-SR), as well as several cell variants of STHE cells in vitro transduced by activated genes (Ha-ras, p53, myc, bcl-2 and neo) and in vivo selected descendant cell variants of all these transformants and transducents were studied. Not one of the in vitro transformed or transduced cell strains were passaged in vivo before the study. Cell strains E-1 and 20/90 represent SV40 in vivo induced primary tumors transferred in tissue culture at less than 20 days after the appearance of the palpable s.c. tumors. All cell strains and their variant lines were cultured as monolayers in vitro with the use of DMEM culture media, adding 5% bovine embryonic serum and antibiotics.

Oncogene-transduction of STHE cells

To make STHE cells susceptible to infection with ecotropic retroviruses, they were transfected with linearized recombinant plasmid containing cDNA for ecotropic receptor under the control of Mo-MuLV LTR promoter (Albritton et al., 1989) and bleomycin resistance gene (bleo) under the control of the SV40 promoter. The retroviral vector carrying cDNA of human bcl-2 (pLBSN) cloned into pLXSN vector was a gift of Dr. P. Neiman (Seattle, WA); cDNA of human c-myc was provided by Dr. R. Eisenman (Seattle, WA) and re-cloned into pLXS vector; retroviral vector pBABE-neo-ras carrying activated v-Ha-ras oncogene was provided by H. Land (London, UK). In all the above vectors, cDNA inserts are expressed under the control of the Mo-MuLV LTR promoter. Biological activity of all these constructs was tested in preliminary experiments, including cooperation with EJ-ras in transformation of primary rat embryo fibroblasts (for p53<sup>175His</sup> and c-myc), suppression of radiation-induced apoptosis in mouse embryo fibroblasts transformed with Ela<sup>+ras</sup> (for p53<sup>175His</sup> and bcl-2, Nikiforov et al., 1996), and transformation of NIH 3T3 cells (for Ha-ras). All the retroviral vectors demonstrated the expected strong effects using the above assays. The stability of the cDNA expression driven by the LTR promoter in hamster cells was tested in STHE cells transduced with pLNCX vector expressing the neo gene from the LTR promoter. These cells maintained stable G418 resistance during 15 passages in vitro and after passing in vivo. Standard procedure of in vivo selection of cell variants was combined with simultaneous quantitative determinations of tumorigenic and spontaneous metastasizing activities (TGA and SMA) (Fig. 1). The procedure used was standardized including (A) the number of tumor cells of the cell strain challenged in normal animals at each cycle of selection; (B) the duration of in vivo s.c. tumor growth. Briefly, after a preliminary in vitro determination of the expression of [H<sub>2</sub>O<sub>2</sub>CA + PGE<sub>5</sub>] phenotype by the parental cells, the first cycle of in vivo selection of each cell strain included the s.c. transplantation of the transformed cells in 4 10-fold differing doses (usually from 2.0 × 10<sup>3</sup> to 2.0 × 10<sup>4</sup>) in the groups of 5 to 6 2 month-old normal Syrian hamsters. All animals in the groups were labeled with individual ear markers and placed in separate cages. After 45–60 days (in most cases at 48–55 days) of s.c. tumor growth, all human c-myc was provided by Dr. R. Eisenman (Seattle, WA) and re-cloned into pLXS vector; retroviral vector pBABE-neo-ras carrying activated v-Ha-ras oncogene was provided by H. Land (London, UK). In all the above vectors, cDNA inserts are expressed under the control of the Mo-MuLV LTR promoter. Biological activity of all these constructs was tested in preliminary experiments, including cooperation with EJ-ras in transformation of primary rat embryo fibroblasts (for p53<sup>175His</sup> and c-myc), suppression of radiation-induced apoptosis in mouse embryo fibroblasts transformed with Ela<sup>+ras</sup> (for p53<sup>175His</sup> and bcl-2, Nikiforov et al., 1996), and transformation of NIH 3T3 cells (for Ha-ras). All the retroviral vectors demonstrated the expected strong effects using the above assays. The stability of the cDNA expression driven by the LTR promoter in hamster cells was tested in STHE cells transduced with pLNCX vector expressing the neo gene from the LTR promoter. These cells maintained stable G418 resistance during 15 passages in vitro and after passing in vivo. Standard procedure of in vivo selection of cell variants was combined with simultaneous quantitative determinations of tumorigenic and spontaneous metastasizing activities (TGA and SMA) (Fig. 1). The procedure used was standardized including (A) the number of tumor cells of the cell strain challenged in normal animals at each cycle of selection; (B) the duration of in vivo s.c. tumor growth. Briefly, after a preliminary in vitro determination of the expression of [H<sub>2</sub>O<sub>2</sub>CA + PGE<sub>5</sub>] phenotype by the parental cells, the first cycle of in vivo selection of each cell strain included the s.c. transplantation of the transformed cells in 4 10-fold differing doses (usually from 2.0 × 10<sup>3</sup> to 2.0 × 10<sup>4</sup>) in the groups of 5 to 6 2 month-old normal Syrian hamsters. All animals in the groups were labeled with individual ear markers and placed in separate cages. After 45–60 days (in most cases at 48–55 days) of s.c. tumor growth, all
animals were sacrificed and the TGAs (expressed in log TrDx) were examined, according to Deichman et al. (1989b). SMA, shown in Tables as (+), (++) or (+++), represents the mean numbers of metastases in the lung tissues of the s.c. challenged group of animals, corresponding to near to 10; to 11–50; or higher than 51 and up to confluent metastatic growth. Cells of 1–3 or more s.c. tumor nodules, each from different animals of the same group, selected by chance, were excised in sterile conditions and cultured as monolayers in vitro. After 4–5 in vitro passages, the cultured tumor cell variants were, as a rule, cleared from host stromal cells and could not be subjected to in vitro determination of the $[H_2O_2]$ phenotype and of TGA and SMA in the subsequent in vivo selection cycle. As the same in vivo selected cell variants were examined for the expression of $[H_2O_2]^{A^+}$ and $[H_2O_2]^{B^+}$ phenotype, and of TGA and of SMA after the completion of the corresponding cycle of selection, the number of cell variants in these challenges, as a rule, coincided, though loss of some variants during in vitro cultivation lead to some alterations in the results shown in the Tables.

Expression of $[H_2O_2]^{C^+}$ and PGE$^S$ phenotype

This was determined according to Deichman et al. (1989b, 1996), including examination of the antioxidant activities of cells and their ability to release PGE$^S$. Tests determining $H_2O_2$-catabolizing activity ($H_2O_2^{CA}$) represent the summarized activity of different cellular enzymatic and non-enzymatic antioxidant mechanisms in their balance with the level of $H_2O_2$-initiated cellular membrane lipid peroxidation chain reaction (LPCR) (for details see Deichman et al., 1996). $H_2O_2^{CA}$ was determined in cell extracts of different cell strains prepared extemporaneously, using luminol-dependent chemiluminescence (LDC); 0.01 M luminol (Serva, Heidelberg, Germany) was used. Cells were extracted in a phosphate buffer, lysing the cells with Triton X-100 at 4°C with subsequent centrifugation (8,000 g, 15 min, 4°C) and determining the protein concentration (Bradford method). Extracts containing 2.0 mg of protein per 1.0 ml (optimal concentration for determination of $H_2O_2^{C^+}$) in a volume of 0.1 ml were treated with 10 mM concentration of $H_2O_2$ (0.1 ml) added with 0.1 ml of luminol and immediately placed at 37°C in thermostated Biolumate (Model 9500, Berthold, Gossenheim, Germany). Two samples of each cell extract ($H_2O_2$-treated), as well as 2 samples of $H_2O_2$ added with phosphate buffer (extract-free control of the rate of $H_2O_2$-inactivation), were used for a comparative parallel determination of LDC. The LDC of such treated cell extracts was registered each 10 sec in the dark, until complete inactivation of $H_2O_2$. The LDC activities of $H_2O_2$-treated cell extracts were expressed as a % of LDC of parallel $H_2O_2$ extract-free control (considered as 100% at the same concentrations of $H_2O_2$, usually actively catabolized $H_2O_2$). The LDC of such treated cell extracts was registered each 10 sec in the dark, until complete inactivation of $H_2O_2$. The LDC activities of $H_2O_2$-treated cell extracts were expressed as a % of LDC of parallel $H_2O_2$ extract-free control (considered as 100% at each time interval). In extracts of cell strains characterized by low levels of catalase and/or of high levels of LPCR, treatment with 10 mM of $H_2O_2$ led to an immediate burst of $O_2$ levels of catalase and/or of high levels of LPCR, treatment with 10 mM of $H_2O_2$ led to an immediate burst of $O_2$, usually exceeding 3 min; for extracts of some cell variants with very low $H_2O_2^{CA}$, it may take more than 120 min.

Determination of PGE$^S$-releasing activity (PGE$^S$)

PGE$^S$ was determined according to Kluchareva et al. (1992) demonstrating that a close 20 min contact between the highly tumorigenic variants of STHE cells and NK cells leads to the rapid (in few min) release of PGE$^S$ into the culture media. PGE$^S$ in the supernatant was determined in parallel by 2 methods: (1) RIA (Young and Knies, 1984); (2) a biological test based on the inhibition of cytotoxic activity (CTA) of fresh intact NK cells by released PGE. The 2 methods gave similar results. The biological test principally used in this study, compared with RIA, is more sensitive, easier to perform, needs no extraction procedure and is highly reproducible (for details see Kluchareva et al., 1992). Briefly, it includes the following steps: (1) gentle centrifugation for 90 sec of the tumor cells/NK cells mixture at a ratio of tumor cells/NK cells of 1:10, with a subsequent 20 min contact period of the mixed cells at 37°C; (2) after another short centrifugation, transfer of cell-free supernatant fluid from cell mixtures (potentially enriched with released PGE) to suspensions of fresh intact NK cells for 20-min exposure at 37°C; (3) routine CTA testing of intact and supernatant-treated NK cells against MOLT-4 cell targets (E:T ratio 20 to 50:1). As controls, culture supernatants from the same intact cells and the cells pretreated with indomethacin (20μl/1.0 ml, 2 hr at 37°C) before contact with PGE-inducing NK cells, as well as intact culture medium, were used. The values obtained for PGE-releasing activity in biological tests represent a relation of CTA of control NK cells to CTA of NK cells treated with supernatants of tumor-NK cell mixtures. These values for PGE-non-releasing cell strains are usually equal to 0.9–1.3. Values equal to, or higher than 1.4, are considered as sensitive, easier to perform, needs no extraction procedure and is highly reproducible positive index of tumor cells PGE-releasing activity (PGE$^S$).
The expression of \([H_2O_2^{CA} + PGE^3]\) phenotype by the cells of the primary SV40 and RSV-SR in vivo induced tumors

In the light of the data presented above, it appeared interesting to examine the possible expression of the \([H_2O_2^{CA} + PGE^3]\) phenotype by the cells of primary tumors induced in vitro by SV40 and by RSV-SR inoculated in newborn Syrian hamsters. Within 20 or fewer days after the appearance of a primary tumor nodule, they were placed in tissue culture and the expression of the \([H_2O_2^{CA} + PGE^3]\) phenotype, of TGA and SMA, were examined. The results shown in Table II demonstrate that there are significant differences in the duration of the latent periods and the numbers of primary tumor nodules between RSV-SR-induced tumors, and the SV40-induced tumors: 8–14 days latent period and the hundreds of primary tumor nodules induced by SV40-transformed cells. However, in contrast to in vitro SV40-transformed cells, both SV40-induced primary tumor cell strains examined at 16–20 days after the appearance of palpable tumor nodules were already expressing the \([H_2O_2^{CA} + PGE^3]\) phenotype, similar to the cells of RSV-SR-induced primary tumor, or RSV-SR-in vitro transformed cells.

In vivo selection of STHE cell lines in vitro transduced with activated genes Ha-ras, myc and p53/175.

Non-selected in vivo parental STHE cells were transduced with activated genes Ha-ras, myc and p53/175 and subjected to in vitro selection in normal animals using the same standard procedure as for virus-transformed cells (above and Material and Methods). The results of the in vivo selection of these cell variants during the s.c. growth of tumors in terms of expression of the \([H_2O_2^{CA} + PGE^3]\) phenotype, TGA and SMA are presented in Table III.

None of these transducents expressed the \([H_2O_2^{CA} + PGE^3]\) phenotype before the in vivo selection, or after the first 2 cycles of selection, but all of them acquired this phenotype after the third in vivo cycle (i.e., about 180 days in vivo). Original levels of TGA of all these transducents determined at the first in vivo challenge varied within limits of 1.0 order, and did not differ in TGA from the parental STHE cells, but during the subsequent second and especially the third cycles of the selection, TGA of Ha-ras and p53/175 transducents increased 30–70 times. A relatively early expression of SMA by some p53/175 transducents was seen already after the second and the third in vivo cycles of selection (Table III).

Thus, the data presented the above indicate that with some individual variations, the dynamics of in vivo selection of cells transformed in vitro spontaneously, or by oncoviruses BAV-3 and SV40, or transduced by Ha-ras, myc and p53/175 are strikingly similar. They all acquired the \([H_2O_2^{CA} + PGE^3]\) phenotype during the third in vivo cycle together with the increase of TGA. In each case, both properties of the \([H_2O_2^{CA} + PGE^3]\) phenotype were acquired in one step: no intermediate cell variants were registered. Some acceleration of SMA by in vivo selected cell variants was observed with SV40 transformed and p53/175-transduced cells.

Delay of in vivo progression of bcl-2 transduced STHE cells

The standard procedure used for the in vivo selection of different in vitro transformants in the case of bcl-2-transduced cells demonstrated the highly delayed rate of in vivo progression, as compared with other cell transformants (see above). In contrast to the latter ones, which acquired the \([H_2O_2^{CA} + PGE^3]\) phenotype during the third in vivo cycle of selection, the in vivo selected variants of bcl-2-transduced remained negative up to the 5th in vivo selection cycle (i.e., during about 280 days) (Table IV). During the same period, there was almost no increase of TGA of selected cell variants [about one order of magnitude in the increase of TGA during 4 cycles (more than 200 days) of in vivo selection]. However, the expression of the \([H_2O_2^{CA} + PGE^3]\) phenotype at the 5th in vivo cycle preceded a significant increase of TGA at the 6th cycle and coincided with the expression of spontaneous metastasis by the majority of cell lines examined at the 5th cycle of selection. At the first and to a slightly lower degree at the second in vivo selection cycles, STHE-bcl-2 tumor nodules were almost free of visible central necrosis, characteristic of the parental STHE cells and of their other transducents. However, at subsequent in vivo selection cycles, the necrotic area of STHE-bcl-2 tumor nodules gradually enlarged and became indistinguishable from the large necrotic area characteristic of the STHE parental tumor nodules.

DISCUSSION

Two main models may be considered in relation to in vivo tumor progression:

(1) A multistep process beginning from the unlimited growth of initiated, or transformed cells, leading to the appearance and to the selection of mutant clones. The clonal nature of the

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**TABLE I** – THE DYNAMICS OF IN VIVO PROGRESSION OF CELLS IN VITRO TRANSFORMED BY BAV-3, SV40, RSV-SR, OR SPONTANEOUSLY

<table>
<thead>
<tr>
<th>Parental cells</th>
<th>In vitro transforming agents</th>
<th>Number of in vivo cycle of selection</th>
<th>Expression of ([H_2O_2^{CA} + PGE^3]) phenotype</th>
<th>TGA [^{1,2}] in log TrD [^{3}] (M ± m)</th>
<th>SMA [^{1,2}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>STHE-C3-neo;</td>
<td>+ neo</td>
<td>1</td>
<td>0/3</td>
<td>3.3–4.6</td>
<td>0/3</td>
</tr>
<tr>
<td>HE-4</td>
<td>BAV-3</td>
<td>0</td>
<td>1/1</td>
<td>&gt;4.3</td>
<td>0/1</td>
</tr>
<tr>
<td>HE-WTSV40</td>
<td>SV40</td>
<td>0</td>
<td>1/1</td>
<td>4.2</td>
<td>0/1</td>
</tr>
<tr>
<td>HET-SR</td>
<td>RSV-SR</td>
<td>0</td>
<td>1/1</td>
<td>0.9 ± 0.4</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>(v-src)</td>
<td>2</td>
<td>3/3</td>
<td>5/5 (+; +; +)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

\[^{1}\]The in vitro expression of \([H_2O_2^{CA} + PGE^3]\) phenotype, and of in vivo TGA and SMA were determined after completion of the corresponding in vivo cycle of selection; \(^{2}\)Numerator: number of cell variants expressing the given property; denominator: number of cell variants examined; \(^{3}\)TGA data (in log TrD \[^{6}\]) are presented either as the minimal and maximal values when n ≤ 3, or as the mean values (M ± m) with n > 3.

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**TABLE II** – THE DYNAMICS OF IN VIVO PROGRESSION OF CELLS IN VITRO TRANSFORMED BY BAV-3, SV40, RSV-SR, OR SPONTANEOUSLY

<table>
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<tr>
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<th>TGA [^{1,2}] in log TrD [^{3}] (M ± m)</th>
<th>SMA [^{1,2}]</th>
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</thead>
<tbody>
<tr>
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<td>0</td>
<td>0/3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>STHE-neo;</td>
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<td>1</td>
<td>0/3</td>
<td>3.3–4.6</td>
<td>0/3</td>
</tr>
<tr>
<td>STHE-C3-neo;</td>
<td>+ neo</td>
<td>2</td>
<td>0/7</td>
<td>2.4 ± 0.2</td>
<td>2/11 (+; +; +++)</td>
</tr>
<tr>
<td></td>
<td>+ neo</td>
<td>3</td>
<td>12/12</td>
<td>2/10 (+; +; +++)</td>
<td>0/3</td>
</tr>
<tr>
<td>HE-4</td>
<td>BAV-3</td>
<td>0</td>
<td>1/1</td>
<td>&gt;4.3</td>
<td>0/1</td>
</tr>
<tr>
<td>HE-WTSV40</td>
<td>SV40</td>
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<td>1/1</td>
<td>4.2</td>
<td>0/1</td>
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<td></td>
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<td>RSV-SR</td>
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<td>1/1</td>
<td>0.9 ± 0.4</td>
<td>0/3</td>
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<tr>
<td></td>
<td>(v-src)</td>
<td>2</td>
<td>3/3</td>
<td>5/5 (+; +; +)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5/5</td>
<td>4/5 (+; +)</td>
<td>4/5</td>
</tr>
</tbody>
</table>

\[^{1}\]The in vitro expression of \([H_2O_2^{CA} + PGE^3]\) phenotype, and of in vivo TGA and SMA were determined after completion of the corresponding in vivo cycle of selection; \(^{2}\)Numerator: number of cell variants expressing the given property; denominator: number of cell variants examined; \(^{3}\)TGA data (in log TrD \[^{6}\]) are presented either as the minimal and maximal values when n ≤ 3, or as the mean values (M ± m) with n > 3.
majority of primary tumors and of spontaneous metastasis suggest that each step of in vivo carcinogenesis is related to a strict selection of extremely rare mutant cell variants, which have the chance to survive and to grow in vivo (Klein, 1955; Fidler and Kripke, 1977; Nowell, 1986). The multistep tumor evolution of spontaneous human tumors may take many years and is related to activation, or/and to suppression of different cell genes (Farber and Cameron, 1980; Klein and Klein, 1984, 1985; Weinberg, 1989; Fearon and Vogelstein, 1990; Field and Spandidos, 1996; Klein, 1996).

(2) However, some types of transformed cells may originally (i.e., without preliminary in vivo selection) express high-grade malignant properties, thus representing “one-step carcinogenesis”; the most striking example being RSV (v-src) in vitro–transformed mammalian cells, which originally (i.e., in the first in vivo passage) demonstrate extremely high tumorigenic and even spontaneous metastasizing activities (Svobodova, 1964). Thus, the 1-step and multistep models of carcinogenesis would appear to differ according to transforming genes, specific mechanisms and phenotypic manifestations.

Our approach to study tumor progression is related to attempts to understand how malignant tumor cells can protect themselves against host macrophages, neutrophils and NK cells. We have thus shown previously that in vivo selected highly tumorigenic variants of STHE cells, in contrast to the parental low-tumorigenic cell variants, are characterized by: (A) A 1- to 2-orders higher resistance to damage induced by H2O2 (a known cytoxic product of activated macrophages and neutrophils) based on high H2O2-catabolizating activity; (B) the simultaneously in vivo acquired ability of the tumor cells to release PGE2 (PGE2) upon contact interactions with NK cells, which leads to rapid inactivation of NK CTA (Deichman et al., 1989b, 1996; Kluchareva et al., 1992). Surprisingly, the same [H2O2 CA + PGE2] phenotype, and extremely high TGA appeared to be expressed in RSV-SR in vitro transformed hamster embryo cells without preliminary selection in vivo (Deichman et al., 1989a).

A non-occasional connection between the expression of [H2O2 CA + PGE2] phenotype and of the cells TGA was demonstrated in our preliminary studies by the use of 2 approaches. (1) The comparison of the expression of the [H2O2 CA + PGE2] phenotype in a large number of STHE cell variants selected in vivo and characterized quantitatively in TGA; it was shown that only highly-tumorigenic cell variants express this phenotype. (2) In experiments, in which antioxidant activity and PGE2 secretion of RSV-SR-transformed cells were inhibited (separately or together) with non-toxic doses of BCNU, indomethacin, or both. Such treatment led to more than 100–150-fold suppression of tumorigenic activity of the cells, (Deichman et al., 1989a,b).

Our present results demonstrate that cell strains in vitro transformed by different oncogenes, though originally negative, would acquire in vivo the expression of the [H2O2 CA + PGE2] phenotype along with increased TGA (Tables I, III, IV). Moreover, all in vivo selected cell variants of in vitro transformed or transduced cells (except bcl-2 transductants), acquired this phenotype at a strikingly similar rate (3 cycles of in vivo selection during about 180 days), i.e., independently of the type of in vitro transforming genes and of the proposed mechanisms of transformation. Strikingly, the transduction of STHE cells with activated genes such as Ha-ras, p533175, myc, and bcl-2 did not influence the level of TGA of these cell lines as compared with the parental cells, although cooperation of transduced genes with unknown oncogene responsible for the spontaneous transformation of these cells, may be implied.

Cell variants expressing the [H2O2 CA + PGE2] phenotype do not apparently preexist in the parental in vitro transduced or transformed cell populations (at least, they have no in vitro selective advantage), but they occasionally appear as rare cell variants in vivo, possibly already at the first cycle of selection, when the total number of cells in the s.c. growing tumor exceed the threshold needed for the appearance of new (mutant) cell variants. The expression of [H2O2 CA + PGE2] phenotype appears to provide the cells with an extremely high in vivo selective advantage.

Two opposed deviations in the rates of acquisition of the [H2O2 CA + PGE2] phenotype by transformed cells are emphasized: as already mentioned, v-src-transformed cells acquire expression of the [H2O2 CA + PGE2] phenotype and a high level of TGA during in vivo selection of these cell variants.
vitro transformation, i.e., at the zero point; as shown in the present study, bcl-2 gene product should provide the transduced cells with a definite selective advantage in vivo. The visible absence of central necrosis in STHE-bcl-2 tumor nodules during the first in vivo cycles (1 and 2) of selection is in line with this expectation; it indicates that very large area of central necrosis, characteristic for the in vivo growth of the parental STHE cells and of its other transductants, is mainly connected with hypoxia-mediated apoptosis, which bcl-2-transduced gene may prevent (Graeber et al., 1996). Two possibilities may be considered in relation to the delay in the progression of bcl-2 tumors. If bcl-2-expressing cells possess higher or equal in vivo selective advantage as the rarely appearing [H2O2CA + PGE3] + bcl-2-expressing cells, the latter have no chance for selection, as the former predominate in the population. In contrast, if the in vivo selective advantages of the rare [H2 O2CA + PGE3] + bcl-2-phenotype-expressing cells are higher than those of bcl-2-expressing cells, the proportion of cells expressing the [H2O2CA + PGE3] phenotype in s.c. tumors would gradually grow (as it is indeed the case). However, a gradual in vivo decrease of bcl-2 expression and thus an increase in the advantages for in vivo selection of [H2O2CA + PGE3]-expressing cells, are not excluded. In such case, expression of the bcl-2 gene apparently competes with expression of the [H2O2CA + PGE3] phenotype and thus apparently counteracts the selection of cells with a [H2 O2CA + PGE3] phenotype and tumor progression.

As shown above, the 30–200-times increased level of TGA of in vivo selected cell variants, coincided as a rule with the expression of the [H2O2CA + PGE3] phenotype (Tables I, III, IV). Possibly, an opposite order of events, i.e., that acquired expression of the [H2 O2CA + PGE3] phenotype promotes the increased ability of tumor cells to survive and to grow in vivo is more likely. In vivo induced primary tumors of SV40 and RSV-SR origin appeared to also express the [H2O2CA + PGE3] phenotype, although they were transferred for in vitro cultivation less than 20 days after their in vivo appearance (Table II). In primary carcinogenesis by SV40 in newborn Syrian hamsters, the appearance and the selection of cells with a [H2O2CA + PGE3] phenotype apparently takes place before the appearance of palpable (as a rule very few) tumor nodules, i.e., during latent periods lasting for 5 to 6 months. The comparison of these values with the 8–12 days latent period of the primary RSV-SR tumors (appearing as hundreds of s.c. nodules induced in newborn Syrian hamsters; Deichman and Kashleva (1987) suggest that expression of the [H2O2CA + PGE3] phenotype corresponds with and possibly determines the duration of the latent periods of the primary carcinogenesis by these viruses.

The expression of [H2O2CA + PGE3] phenotype and of a high level of TGA in RSV-SR transformed cells has been shown to be related to v-src activity (Deichman et al., 1996). The acquired expression of the same phenotype by in vivo selected malignant variants of tumor cells transformed in vitro by oncogenes unrelated to the v-src family may reveal the existence of a common c-src-related mechanism of expression of this phenotype; among many possibilities, a potential role of SH2 and/or SH3 activities (Hirai and Warmu, 1990) in such cell variants may be proposed. In this case, the v-src-induced one-step carcinogenesis and the multistep carcinogenesis originated by different cell-transforming genes appears to be strikingly similar in some discrete secondary phenotypic manifestations, corresponding with levels of TGA and with rate of in vivo tumor evolution. The acquired expression of the [H2O2CA + PGE3] phenotype in in vivo selected malignant tumor cell variants of different origin raise questions on how such stable phenotype is maintained and how it co-exists with numerous individual tumor cell characteristics, which may be independently acquired via activation of particular oncogenes.

The rate of in vivo acquisition of spontaneous metastasizing properties by SV40-transformed and by p53,715-transduced cell variants was higher, as compared to all other in vitro transformed cell strains (Tables I, III, IV). Some of such highly metastatic cell variants have already been noted at the second in vivo cycle of selection. We already noticed in few observations (data not shown), that the expression of the [H2O2CA + PGE3] phenotype by the cells of spontaneous metastases disseminated from s.c. tumor nodules, may forestall the expression of [H2O2CA + PGE3] phenotype by the cells of s.c. tumors (which become detectable only when such cells predominate in a s.c. tumor). Regardless, the suppression of wild type p53 in the cells by LT54V- and by mutant p53,715 may be responsible for this accelerating effect. The data presented (Table I) on the in vivo selection of spontaneously metastasizing variants of non-metastasizing HET-SR cells indicate that acquisition of the [H2 O2CA + PGE3] phenotype along with maximal extent of TGA and of SMA, are apparently separate steps in tumor progression.

Thus, the expression of the [H2O2CA + PGE3] phenotype and of increased TGA in all in vivo selected cell variants of cell strains in vitro transformed by different oncogenes, suggests that there are discrete differences between in vitro transformed cells and their in vivo selected descendants: a gradual in vivo replacement of the parent cells by the latter ones necessarily takes place, sooner or later. The expression of the [H2O2CA + PGE3] phenotype in tumor cells apparently represent the second discrete genetic event in tumor progression, that occurs following transformation. These 2 events are not separable in the case of v-src transformation, indicating that at least some molecular mechanisms of tumor malignancy are hidden in in vitro v-src-transformed cells. The in vivo acquired ability of malignant variants of tumor cells expressing the [H2O2CA + PGE3] phenotype to protect themselves against oxygen stress and some host anti-tumor effectors, emphasizes the direction and the host-dependence of an apparently essential mechanism in the natural evolution of tumors in vivo.

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