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Invited Review

Elimination of transformed cells by normal cells: a novel concept for the control of carcinogenesis

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Summary. Control of transformed cells by neighbouring normal cells is known since the beginning of transformation studies in vitro. The classical explanation for this phenomenon is based on proliferation inhibition of transformed cells by normal cells. We extend this model by presenting data that show that TGF-ß-treated normal cells can eliminate transformed cells by induction of apoptosis. Both the TGF-ß-induced signal pathway in normal cells, leading to the production of a short-lived apoptosis-inducing factor, as well as the specific interaction of this factor with transformed cells depend on the action of reactive oxygen species. Sensitivity to induction of apoptosis seems to be a common feature associated with the transformed state, independent of the originally transforming principle. Therefore, tumor development should require either interference with the process of elimination or acquisition of resistance against it. We discuss experimental evidence for interfering substances, such as antioxidants, as well as for genetic systems that protect transformed cells from the negative effects of their cellular environment, such as Bcl-2 or papilloma viruses. These findings, as well as the general resistance of ex-vivo tumor cells against induction of apoptosis are in line with the novel model of control of tumor progression presented by us in this review.

Key words: Apoptosis, Antioxidants, Carcinogenesis, Inhibition, Proliferation, Reactive oxygen species, Transformed cell, Tumor promoter, TGF-beta

I. The classical concept of transformed cell inhibition by normal cells

Inhibition of transformed cells by neighbouring normal cells has been known for as long as transformation studies have been performed in vitro. Berwald and Sachs (1963) stated in their classical paper that «transformed cells could be inhibited from forming clones when grown together with normal cells in confluent monolayer». Stoker was the first to directly demonstrate inhibition of proliferation of transformed cells by normal cells (Stoker, 1964, 1967; Stoker et al., 1966). This work has been confirmed and extended by many groups and thus has established the classical concept to explain the negative interaction between normal and transformed cells (Borek and Sachs, 1966; Macintyre and Ponten, 1967; Ponten and Macintyre, 1968; Weiss, 1970; Bignami et al., 1988a,b). The need for direct contact between normal and transformed cells for effective inhibition was confirmed by several investigators. Cell density and serum concentration were shown to play a crucial role in this process (Bertram, 1977; Bertram et al., 1982). Modulation of the cAMP level with its subsequent influences on the degree of inhibition indicated an essential involvement of direct intercellular contacts (Bertram, 1979; Bertram et al., 1982; Bertram and Faletto, 1985). Based on the work of Loewenstein (Loewenstein, 1979), Mehta et al. (1986) showed that gap junctional communication was essential for inhibition of transformed cell proliferation by normal cells. Martin et al. (1991) presented evidence of an inhibitory mechanism that was independent of direct intercellular contact, but was mediated by secreted factors.

La Rocca et al. (1989) established a different model for the control of transformed cells by their normal counterparts. They showed that normal cells induce differentiation of cells transformed by the myc oncogene. In addition, Ramon y Cajal et al. (1994) recently suggested that this mechanism is responsible to for the inhibitory effect of dermal fibroblasts on keratinocytes transformed by the ras oncogene, which has been originally described by Dotto et al. (1988) and Missero et al. (1991). In this system, a secreted factor, most likely TGF-ß3, seems to play a crucial role. Interestingly, transformed cells expressing the adeno-virus E1A gene or papilloma-derived ras-transformed keratinocytes were resistant against induction of tumor suppression (Misero et al., 1991; Ramon y Cajal et al., 1994), pointing to a defined and crucial role of DNA

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viruses during skin carcinogenesis.

There are numerous reports on the negative influence of high numbers of normal cells on the efficiency of transformation by chemical or physical carcinogens and defined oncogenes, as well as on spontaneous transformation (Reznikoff et al., 1973; Haber et al., 1977; Han and Elkind, 1979; Terzaghi and Little, 1976; Kennedy et al., 1980; Fernandez et al., 1980; Mordan et al., 1983; Spandidos and Wilkie, 1984; Land, 1986; Land et al., 1986; Dotto et al., 1989, 1989a; Kakkanas and Spandidos, 1990; Farber and Rubin, 1991). Several attempts have been made to optimize transformation systems in order to minimize the negative effects exerted by normal cells. Usually the critical parameters were the density of the normal cells and the size of the potentially transformed clone at the time of confluence of the culture (Reznikoff et al., 1973; Bertram, 1977; Harber et al., 1977; Mordan et al., 1983; Schechtman et al., 1987). Spandidos and Wilkie (1984), as well as Land et al. (1986) demonstrated that the oncogenic potential of the ras oncogene was sufficient for transformation. However, transformation was only measurable when the normal cells surrounding oncogene-bearing cells were killed. Alternatively, transfection of the majority of cells in a population of normal cells with an oncogene-containing retroviral vector resulted in efficient transformation of the majority of the cells and in this way led to a suboptimal density of remaining normal cells (Land, 1986). Under these conditions, transformation was readily detectable. These finding led Weinberg to formulate that «the growth properties of a cell depend not only on its own genotype (e.g. its complement of oncogenes) but on its environment as well» (Weinberg, 1989).

II. Interference with growth control by tumor promoters

Based on the established concept that proliferation of transformed cells is inhibited by normal cells via direct gap junctional interaction (Mehta et al., 1986), and on the finding that tumor promoters are able to interfere with gap junctional communication (Murray and Fitzgerald, 1979; Yotti et al., 1979; Guy et al., 1981; Newbold and Amos, 1981; Enomoto and Yamasaki, 1985), several groups established a concept that involved interference of tumor promoters with growth control of transformed (initiated) cells (Mordan et al., 1982; Frazelle et al., 1983; Trosko and Chang, 1984, 1986; Dotto et al., 1985; Kanno, 1985; Loch-Caruso and Trosko, 1985; Rivedal et al., 1985; Yamasaki et al., 1985; Binggeli and Weinstein, 1986; Herschman and Brankow, 1986, 1987; Boreiko et al., 1987; Frixen and Yamasaki, 1987, 1988, Fitzgerald and Yamasaki, 1990; Klaunig and Ruch, 1990; Trosko et al., 1990). This model implies that initiation is sufficient to induce the transformed state of cells. Tumor promoters interfere with the direct inhibitory effect exerted by normal cells and by constraining growth control, they allow focus formation. This concept has been shown to be applicable for cells transformed by UV light, chemical carcinogens or defined oncogenes. In accordance with this concept, enhancement of gap junctional communication has been shown to inhibit measurable transformation (Pung et al., 1993; Hossain et al., 1989). The oncogene ras seems to be able to induce a similar effect as the tumor promoter TPA (Dotto et al., 1989b; el-Fouly et al., 1989), implying that ras is involved in the transformation of cells as well as in interference with growth control by neighbouring normal cells. However, there is emerging evidence that the concept of tumor promotion via inhibition of intercellular communication is not generally applicable. No direct correlation between inhibition of gap junctional communication and influence on focus formation in some experimental systems has been observed (Boreiko et al., 1989; Husoy et al., 1993; Mikalsen and Sanner, 1993). Dorman et al. (1983) showed that the concentration of TPA required for tumor promotion was different from that required for inhibition of gap junctional communication. There was also early evidence that provision of growth advantages was not sufficient to explain the complex process of tumor promotion. It has been mainly the work of Colburn et al. (1979, 1980) that has indicated that tumor promotion also requires induction of stable changes in cells. Thus, initiation alone does not seem to be sufficient for complete transformation. Incomplete transformation by the initiator, induction of additional qualitative changes by the tumor promoter and lack of provision of selective growth advantages for transformed cells by the tumor promoter, were directly demonstrated for the tumor promoting activity of TGF-ß (Bauer et al., 1991). In contrast, Hamel et al. (1988) speculated that the tumor promoting effect of TGF-ß was due to the establishment of selective growth advantages (Hamel et al., 1988). This assumption is in direct discrepancy to our findings and to the results of subsequent reconstitution experiments by us (Bauer et al., 1991; Höfler et al., 1993) and by Silingardi et al. (1994).

III. A novel concept: induction of apoptosis in transformed cells by TGF-ß-treated normal cells

The existing concepts of inhibition of transformed cells by normal cells depend on the inhibition of proliferation (i.e. control of cell number) or induction of differentiation (i.e. control of the transformed phenotype). Both models, however, imply survival of transformed cells. In addition to these classical concepts, we have demonstrated a novel concept which is based on elimination of transformed cells by normal cells through induction of apoptosis. It requires the presence and activity of normal cells and is triggered by TGF-ß.

The initial finding leading to this novel concept started during the study of TGF-ß-mediated tumor promotion (Bauer et al., 1991). TGF-ß is a member of a large family of highly active, pluripotent cytokines. The classical activity of TGF-ß comprises its ability to

induce reversible colony formation (morphological transformation) of fibroblasts when it is administered together with epidermal growth factor (Moses et al., 1981; Roberts et al., 1981, 1982). Colony formation in soft agar is paralleled by criss-cross morphology in monolayer. Both features of the transformed state disappear after removal or exhaustion of the growth factors in the system. Meanwhile, many other activities of TGF-ß have been characterized (for review see: Massagué, 1987, 1990, 1992; Roberts and Sporn, 1988, 1990; Barnard et al., 1990; Strauß et al., 1995): stimulation or inhibition of cellular growth, induction or inhibition of differentiation, induction of synthesis of extracellular matrix and negative interference with the immune system. Inactive («latent») high molecular weight TGF-ß was first described in serum by its ability to activate latent Epstein-Barr virus (EBV) antigen synthesis (Bauer et al., 1982a,b,c). Conformational changes due to pH shock converted the inactive factor into its active form. The identity of the EBV inducing factor as TGF-ß was determined after sequence analysis (Bauer, unpublished result; Strauß et al., 1995). TGF-ß can stably transform rat fibroblasts (Vossbeck et al., 1995). It acts also as a potent tumor promoter (Bauer and Höfler, 1985; Hamel et al., 1988; Marks et al., 1988; Fürstenberger et al., 1989; Sieweke et al., 1990; Bauer et al., 1991). Cells transformed directly or during initiation/promotion maintain their transformed state via an autocrine TGF-ß loop (Wehrle et al., 1994). Tumor promotion experiments stringently require low cell density at the beginning. During initiation/promotion experiments, both the initiator and the promoter induce qualitative changes in the cells, leading finally to the transformed state. To test for additional potential induction of selective growth advantages for transformed cells surrounded by their parental normal cells, we reseeded mixtures of normal cells and foci of transformed cells, both in the absence and presence of TGF-ß (Bauer et al., 1991). According to the concept of provision for selective growth advantages by tumor promoters, we expected an increase both in number and size of appearing foci. We were rather surprised to see the opposite: TGF-ß completely inhibited focus formation in the mixture of normal and transformed cells. Reconstitution experiments, using stably transformed fibroblasts and normal fibroblasts of different passage numbers confirmed this finding (Höfler et al., 1993): coculture in the presence of TGF-ß reduced both the number of transformed cell foci and the number of colonies detectable after seeding in soft agar. In the absence of TGF-ß, normal cells also suppressed transformed cells, though to a lesser degree than in its presence. This finding shows that normal cells, in the absence of exogenous TGF-ß, exert a basal eliminative activity, which increases with their passage number. Abrogation of this basal activity by anti-TGF-ß revealed that endogenous TGF-ß was involved in its induction. Reconstitution experiments also confirmed that the inhibitory effect was dependent on the concentration of TGF-ß. TGF-ß exhibited its inhibitory effects on

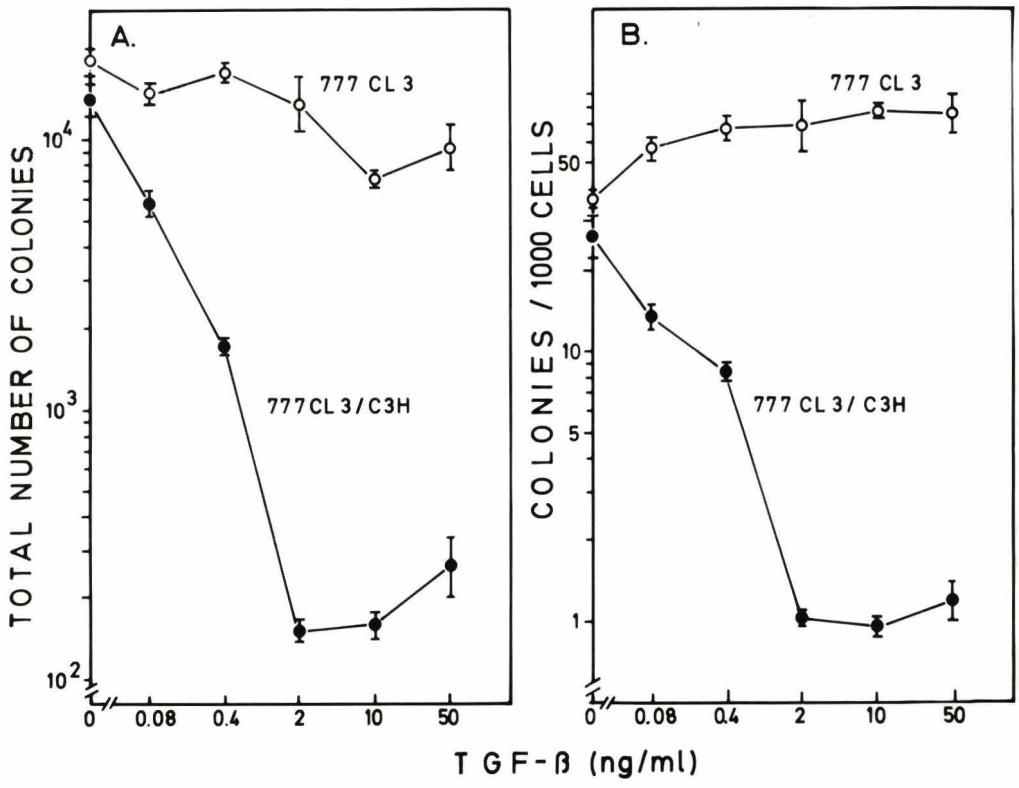
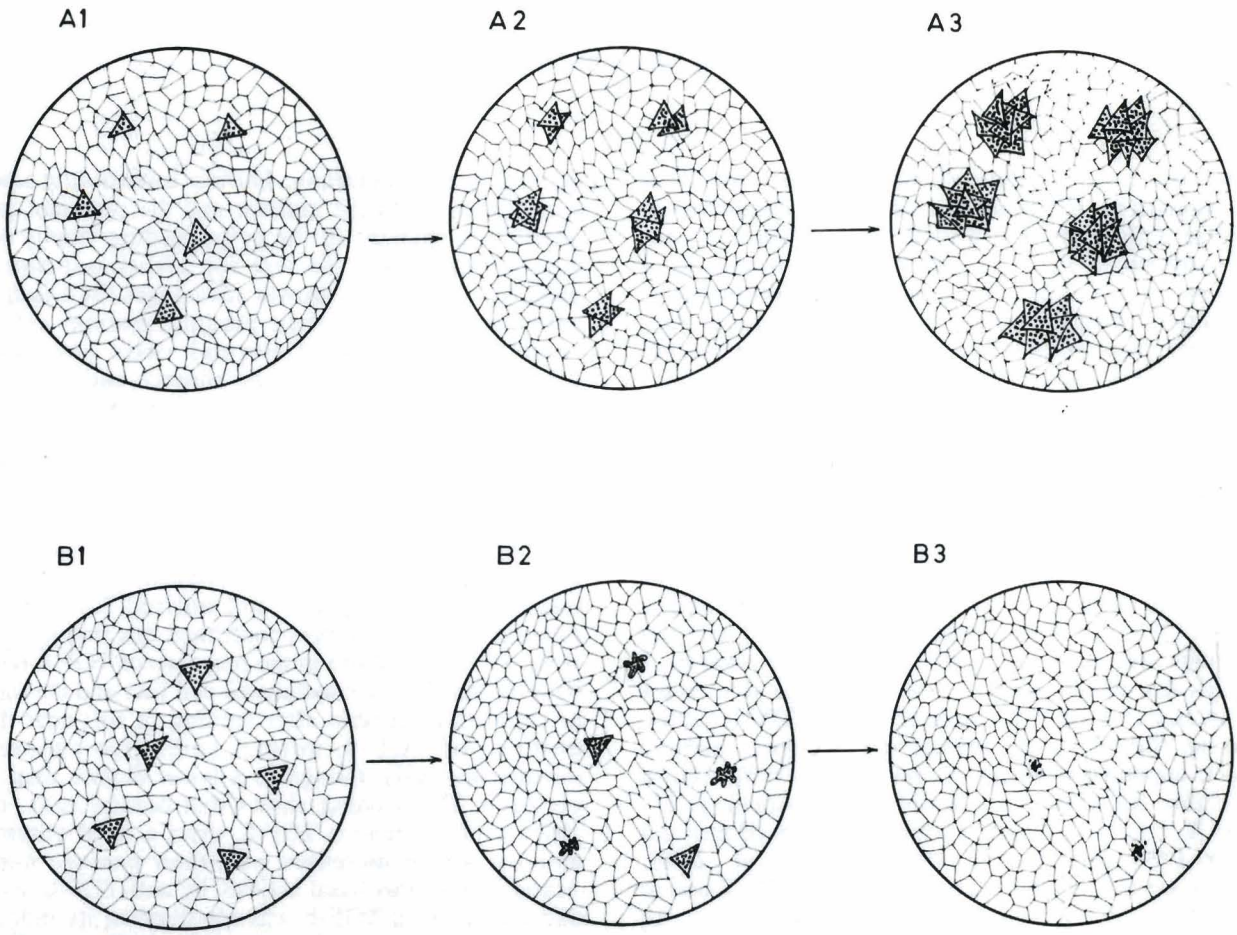


Fig. 1. Inhibition of transformed cells depends on the concentration of TGF-ß and on the presence of normal cells. 2000 transformed 777 Cl3 cells were cultivated in the absence or presence of 20000 normal C3H10T1/2 mouse fibroblasts and the indicated concentrations of TGF-ß1 in Costar 6 well tissue clusters. After one week, the assays were trypsinized and 20000 cells from each assay were seeded into soft agar. After 10 days, the number of colonies (larger than 20 cells/colony) were determined. A: total number of colonies; B: colonies/10000 cells. 777 Cl13 cells were obtained after initiation of C3H 10T1/2 cells with UV light and tumor promotion with TGF-ß (Bauer et al., 1991; Wehrle et al., 1994). The experiments show that TGF-ß has no direct inhibitory effect on transformed cells. In the presence of normal cells, however, there is a massive decrease of colony forming transformed cells dependent on the concentration of TGF-ß. These data have been taken from Höfler et al., 1993.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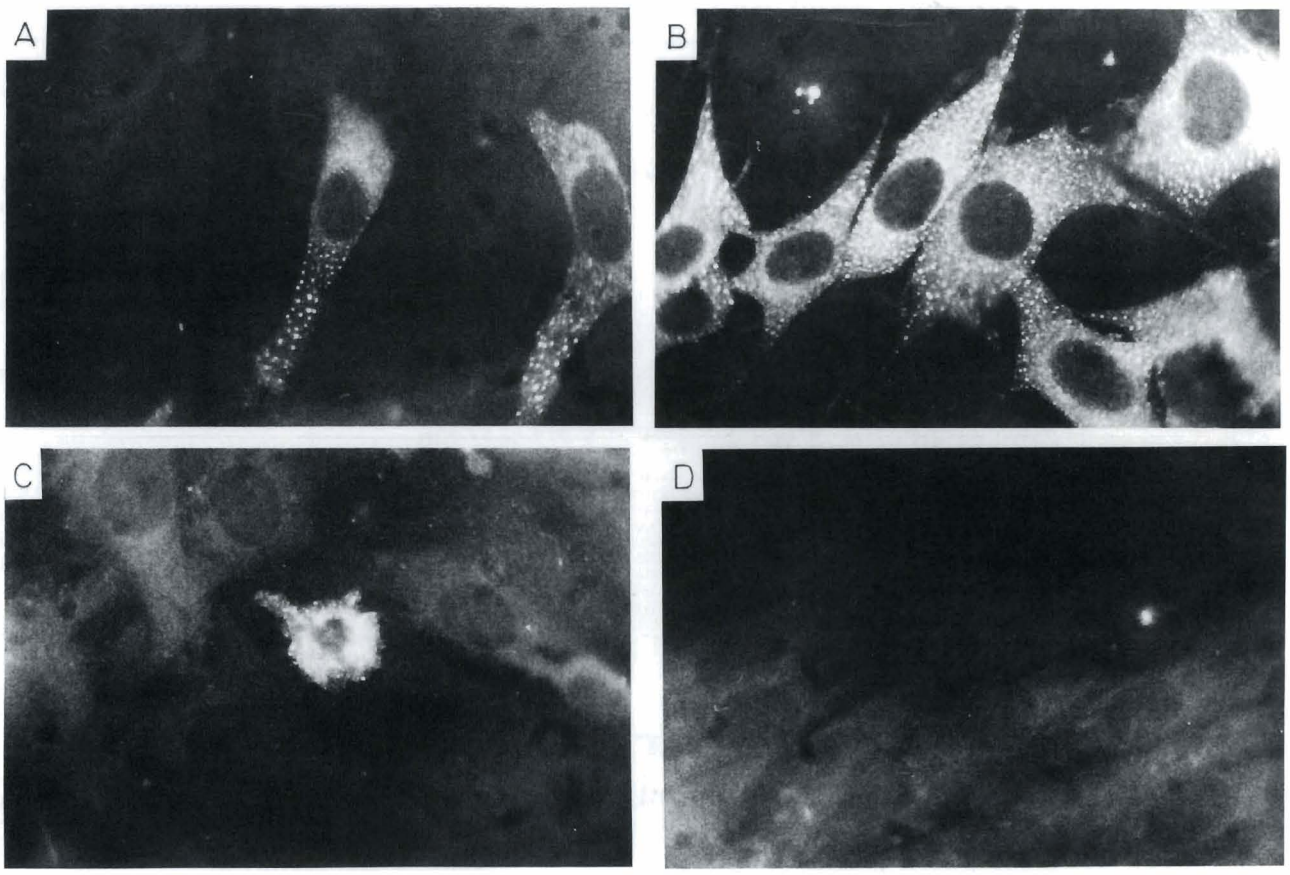
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transformed cells not directly, but only in the presence of sufficient numbers of normal cells (Fig. 1).

The drastic reduction in the number of colony-forming cells after coculture with normal cells and TGF-ß might have theoretically been explained by different mechanisms, such as reversible or irreversible inhibition of transformation, reversion to the normal phenotype, induction of the postmitotic state or elimination. The use of transformed cells that were resistant to G 418 and that expressed the MxA antigen enabled us to demonstrate that the major cause of transformed cell loss in coculture with normal cells and TGF-ß was due to their elimination (Jürgensmeier et al., 1994a). The most convincing evidence was obtained by a direct follow-up of transformed cells, as pointed out in Figs. 2, 3. Spontaneously transformed cells were isolated from a culture of Swiss 3T3 cells that expressed the MxA gene, a gene confering resistance against orthomyxovirus (Pavlovic et al., 1990). The MxA antigen gives a characteristic staining in indirect immunofluorescence and thus allows detection of individual MxA-expressing transformed cells (Fig. 2). Normal cells and MxA-expressing transformed cells were cocultured in the presence and absence of TGF-ß. After attachment of the cells, and at intervals of two days, cocultures were fixed and stained for MxA antigen-positive cells. At the beginning of the experiment, individual transformed cells were detected. This finding is of central importance for the following argumentation, as it demonstrates that the sensitivity is high enough to detect single cells. Cocultures without TGF-ß showed that most of the originally seeded transformed cells formed clones in which the number of cells increased with time. The total number of clones corresponded to the number of single cells at the beginning of the experiment. In cocultures in the presence of TGF-ß two major observations were made: two days after the start of the experiment, most of the initially seeded cells were still detectable. However they had proliferated less efficiently than cells in the control assay. This points to a control of proliferation. However on day four, the number of total clones in cocultures with TGF-ß was drastically reduced. In the assays with TGF-ß, apoptotic cells were detected as characterized by membrane blebbing and chromatin condensation. This finding demonstrates a massive elimination of transformed cells. Fig. 3 shows the quantitation of the result demonstrated in Fig. 2.

Separation of transformed and normal cells, using tissue culture inserts gave further evidence of specific elimination of transformed cells after coculture with spatially separated TGF-ß-treated normal cells (Jürgensmeier et al., 1994b). This finding also demonstrates that direct cell-to-cell contact is not necessary for induction of cell death. The requirement for high densities of normal cells therefore reflects the need for a large concentration of effector cells rather than the necessity for direct contact of normal and transformed cells for induction of cell death.

The use of tissue culture inserts enabled the separate study of the effects on transformed and normal cells. Under optimal conditions, the complete population of

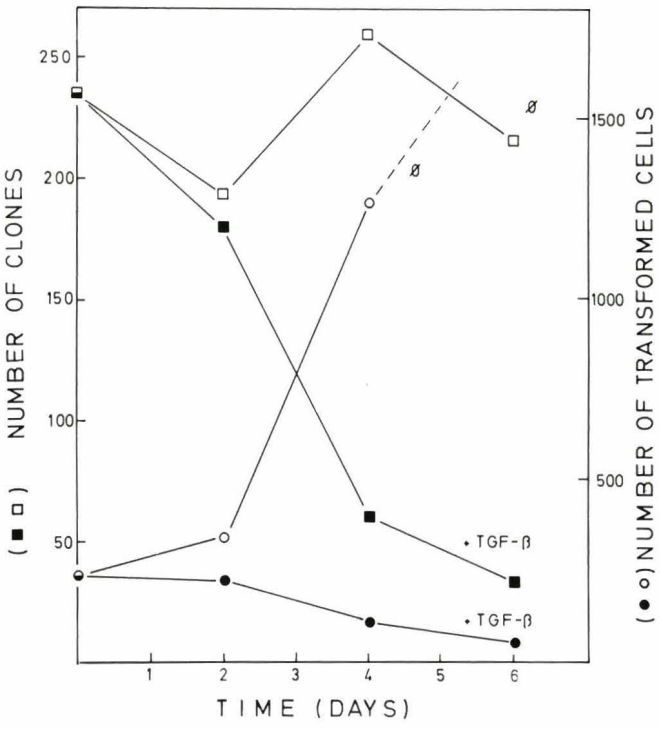


Fig. 3. Quantitation of transformed MxCl1 cell elimination by normal cells and TGF-ß. The experiment was performed as described in Figure 2. Transformed MxCl1 cells (240 cells/assay) were seeded together with 40000 normal C3H 10T1/2 cells in the absence or presence of 10 ng/ml TGF-ß1. At the indicated times assays were stained for MxA expression using indirect immunofluorescence. The number of clones as well as the total number of cells were determined. The graph shows that coculture in the absence of TGF-ß allows all seeded cells to form clones with increasing numbers of cells over time (Due to the low passage number of normal cells used in this assay, there was no basal inhibitory activity). In the presence of TGF-ß, coculture leads to a loss of the majority of the cells initially seeded. Only a few clones with a small number of cells survive. This experiment therefore directly demonstrates elimination of transformed cells by normal cells and TGF-ß.

Fig. 2. Direct demonstration of transformed cell elimination by normal cells and TGF-ß. The upper part of the figure summarizes the rationale of our experiment. Small numbers of transformed MxCl1 cells (dotted triangles) were seeded together with excess normal cells in the absence (A1-3) or presence of TGF-ß (B1-3). At days 0 (A1/B1), 2 (A2/B2) and 6 (A3/B3) assays were stained for MxA-expressing transformed cells using indirect immunofluorescence. Individual transformed cells can be detected after seeding (day 0). In control assays, cells form clones with increasing cell number. In TGF-ß-containing assays inhibition of proliferation and then induction of apoptosis of transformed cells is seen. At day 6 most of the transformed cells are eliminated. The lower part of the figure demonstrates transformed MxCl1 cells, stained for MxA expression. A: individual cells; B: a clone of proliferating intact MxCl1 cells; C: apoptotic MxCl1 cells; D: layer of normal cells free of transformed cells. The quantitative analysis of this approach will be shown in Figure 3.

transformed cells died during the experiment. In other experiments, a large number of cells died, whereas others survived and continued to proliferate. These opposite effects could lead to a net balance of surviving cells which would not easily allow quantitation of ongoing transformed cell elimination. This problem can be overcome by clonal analysis of transformed cells. Transformed cells were seeded sparsely in tissue culture clusters and allowed to grow to clones consisting of 25-50 cells per clone. Then tissue culture inserts containing TGF-ß-pretreated normal cells were transfered and cocultured. The more sensitive clones were the first to show signs of apoptosis and massive cell death, while the less sensitive clones continued to proliferate and showed cell death later. The dissociation of the random population of cells into individual clones thus enabled study of the induction of apoptosis independently from the proliferation of less sensitive clones (Langer et al., 1995).

To study the nature of transformed cell death, morphology, chromatin structure and DNA integrity were determined after coculture with TGF-ß-treated normal cells. Transformed rat cells cocultured with normal cells in the presence of TGF-ß showed distinct signs of apoptosis (Jürgensmeier et al., 1994b): prominent membrane blebbing, condensation of chromatin and fragmentation of DNA. Apoptotic transformed mouse cells showed membrane blebbing and chromatin condensation (Fig. 4), but no DNA fragmentation. Lack of DNA fragmentation into small fragments during apoptosis of mouse cells has been originally reported by Tomei et al. (1993). These data show that transformed cells in coculture with normal cells and TGF-ß are not unspecifically damaged, but are rather induced to undergo an active program of apoptosis. Apoptosis can be induced by radiation, certain chemicals, reactive oxygen and in certain cell types by TGF-ß or TNF directly (Kerr and Harmon, 1991). In our system, TGF-ß seems to induce normal cells to release apoptosis-inducing signals that act specifically on transformed cells (Langer et al., 1995). Attempts to transmit the activity by supernatants or to concentrate it have regularly failed. This points to a short-lived factor that can only result in induction of apoptosis if it is transmitted between normal and transformed cells immediately.

Sensitivity of transformed cell lines

Cells transformed in vitro were generally sensitive to induction of elimination by TGF-ß-treated normal cells, irrespective of the nature of the agent that originally caused their transformation (Jürgensmeier et al., 1994a). Thus, cells transformed by TGF-ß, UV and TGF-ß, Rous sarcoma virus, Moloney sarcoma virus, HSV or spontaneously, showed the same phenotype. Recent experiments include cells transformed by a variety of oncogenes (Bauer et al., in preparation) and cells transformed by chemical carcinogens (Panse et al., in preparation). Thus, sensitivity against induction of apoptosis seems to be either a general feature of the acquired transformed phenotype, or alternatively, a prerequisite for transformation. The only exception found in this context is represented by cells transformed by bovine papilloma virus (Melchinger et al., in preparation). These were regularly found to be resistant, indicating that papilloma virus infection protects transformed cells from elimination; a concept whose impact will be discussed later in more detail. In contrast to in vitro transformed cell lines, ex vivo tumor lines were generally found to be resistant to induction of apoptosis (Engel unpublished results; Brauns, unpublished result). One possible interpretation of this finding is that resistant transformed cells were selected during tumor cell progression. Tumor induction experiments in nude mice are presently being performed to directly address this question.

Ability of normal cells to induce apoptosis after TGF-ß treatment

The ability of fibroblast lines and non-immortalized fibroblast cultures of different species, including man, to induce apoptosis in transformed cells after treatment with TGF-ß has been observed. A clonal assay system was established to clarify whether a distinct subpopulation of fibroblasts or all cells within the population were able to induce apoptosis (Fig. 5). Normal cells were sparsely seeded and allowed to grow into clones, which were marked and overlaid with an excess of transformed cells, in the presence or absence of TGF-ß. After four to five days of coculture, induction of apoptosis could be readily measured. In assays containing optimal concentrations of TGF-ß, all clones of normal cells exhibited induction of apoptosis of transformed cells. This finding indicates that all cells and not just a specialized subpopulation have this biochemical activity. In the absence of exogenous TGF-ß, the vast majority of clones of normal cells showed overlaying undamaged transformed cells. A few clones, however, showed massive elimination of transformed cells. Subsequent assays with suboptimal concentrations of TGF-ß showed that, though all the clones are able to induce apoptosis, the threshold level for TGF-ß to induce production of apoptosis-inducing factors is different from clone to clone (Picht et al., 1995). Clones eliminating in the absence of exogenous TGF-ß seem to represent the most sensitive ones which are induced by TGF-ß released from transformed cells themselves. Their activity explains the «basal eliminative activity» seen in cocultures of normal and transformed cells in the absence of exogenous TGF-ß.

Triggering of elimination by transformed cells themselves

TGF-ß is released by many types of transformed cell lines, irrespective of the original transformation principle (Moses et al., 1981; Roberts et al., 1982;

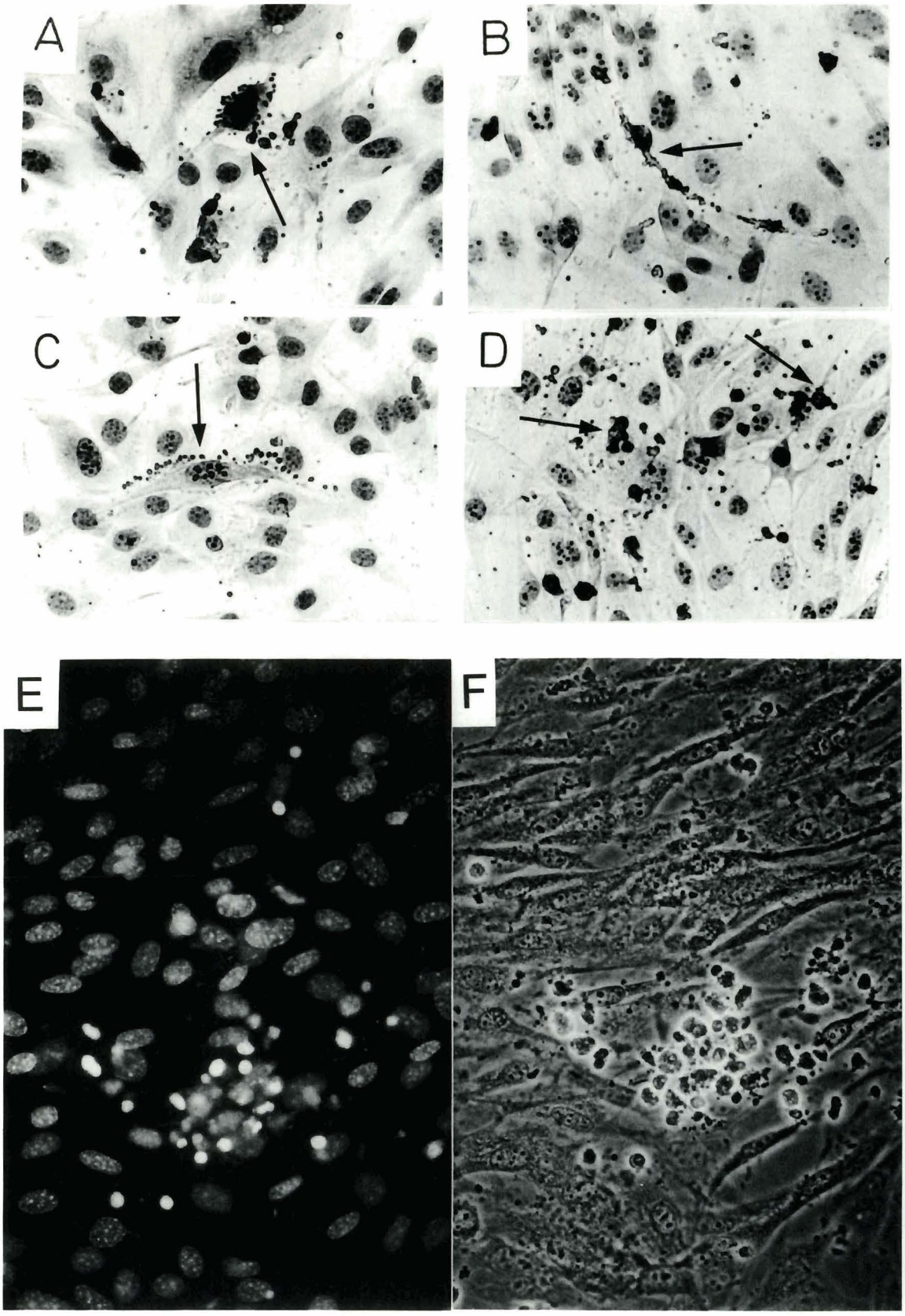
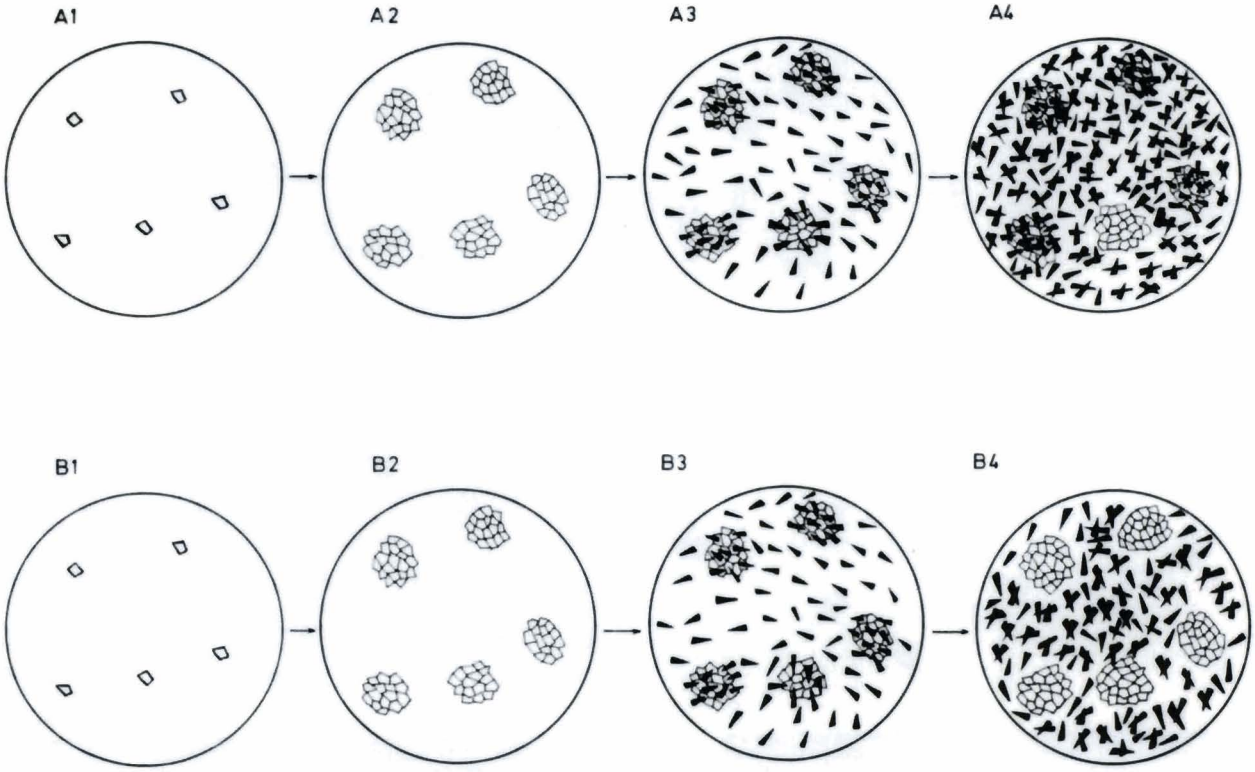


Fig. 4. Elimination of transformed cells by normal cells and TGF-ß is due to the induction of apoptosis. Normal C3H10T1/2 cells were cocultivated with transformed MxCl1 cells in the presence of TGF-ß, as described in Figure 2 and 3. **A-D**. At day 5, assays were fixed with ethanol and stained with Giemsa's solution. Arrows indicate apoptotic transformed cells, characterized by membrane blebbing and chromatin condensation. **E, F**. Assays were fixed with methanol on day 4 after coculture. In staining with Hoechst dye; in **F** the same focus is shown by phase contrast microscopy. Note that the transformed cells show chromatin condensation and morphological alterations as a sign of apoptosis, whereas normal cells remain intact. These data have been taken from Jürgensmeier et al., 1994a.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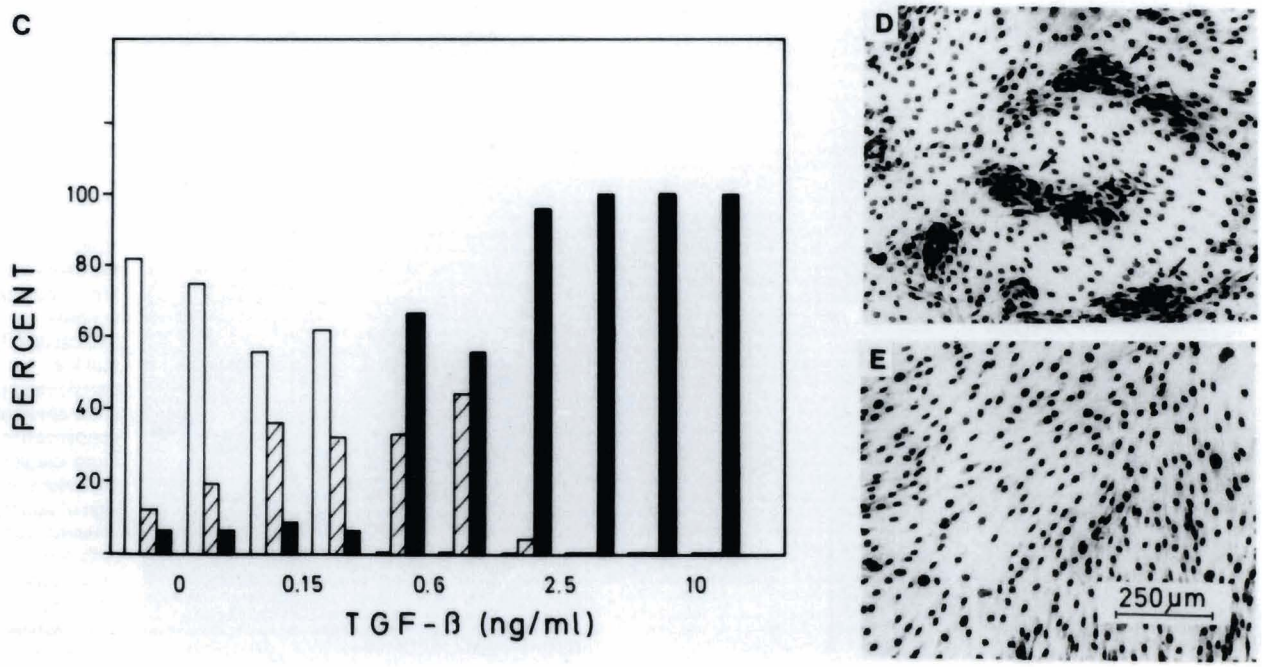
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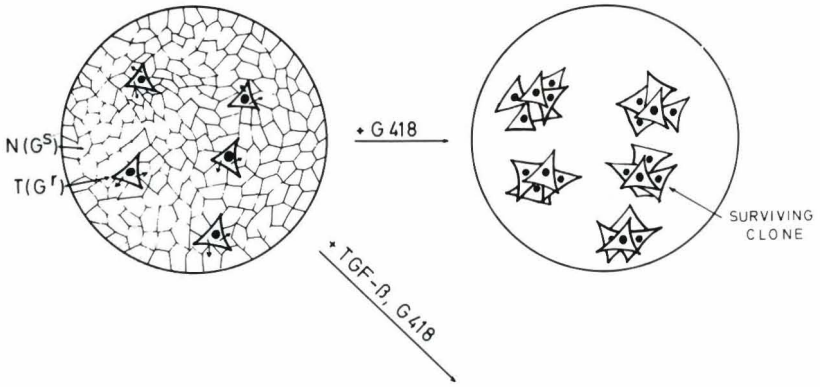
Anzano et al., 1983; Nickel et al., 1983; Tucker et al., 1983; Massagué, 1984; Derynck et al., 1985, 1987; Goustin et al., 1986; Keski-Oja et al., 1987). Based on the concept of autocrine stimulation by growth factors as originally proposed by Temin (1967) and extended by Sporn and Todaro (1980), as well as by Sporn and Roberts (1985), functional involvement of TGF-ß released by transformed cells in the maintenance of their transformed state via an autocrine loop has been suggested by many investigators (for review see Goustin et al., 1986). It has been explicitely demonstrated for the cell lines used in our studies (Wehrle et al., 1994). Coculture experiments with increasing numbers of transformed cells show, that these can trigger induction of their own apoptosis in a TGF-ß-dependent process (Hackenjos et al., 1995). This effect can be measured in a quantitative way by the use of relatively small numbers of G418-resistant transformed indicator cells (whose survival could easily and quantitatively be monitored), excess normal effector cells and G418-sensitive transformed inductor cells (Fig. 6). An increase in the number of inductor cells decreased the number of surviving indicator cells. Induction was abrogated by anti-TGF-ß. This assay shows that transformed cells can trigger induction of their own apoptosis, provided they are present in sufficient concentration. The need for large cell numbers seems to be a specific requirement of the in vitro system, where the vast excess of medium above the thin monolayer of cells dilutes effector molecules efficiently. It is reasonable to assume that under the conditions of close cellular vicinity in the in vivo situation, TGF-ß released from a newly transformed cell surrounded by normal cells will be sufficient for induction of elimination, as there is no dilution of the cytokine. This scenario describes an interesting dual role of TGF-ß during carcinogenesis. Its procarcinogenic activity for induction of transformation and maintenance of the transformed state is opposed by its ability to trigger negative effects of normal cells against transformed cells. Experimental dissection in vitro of inductor and indicator cells allows measurement of the inducing activity of different cell lines irrespective of their own sensitivity. In combination with neutralizing antibodies against TGF-ß, it can be used as a sensitive and specific means to measure release of active TGF-ß.

Mechanism of transformed cell elimination by TGF-ß-treated normal cells

After defining the essential cell biological parameters of apoptosis induction in transformed cells by normal cells, attempts to define the biochemical mechanisms involved have been made. Dissection of the system into experimentally accessible distinct steps (Jürgensmeier et al., 1994b) and using the sensitivity of the clonal analysis of induction of apoptosis has shed light on several interesting points (Langer et al., 1995). First of all, induction of apoptosis was found not to be a direct effect of TGF-ß on transformed cells. Neither was it mediated by TNF, a potentially interesting candidate for the activity observed. Furthermore, the whole process can be divided into a TGF-ß-dependent step and subsequent TGF-ß-independent steps. Induction of the production and release of apoptosis-inducing factors from normal cells is the only TGF-ß-dependent process in this system. If induced by exogenous TGF-ß, it does not require the presence of transformed cells. The essential and critical signal is TGF-ß. It induces production of apoptosis-inducing signal molecules that is continued after TGF-ß is washed away and neutralizing antibodies against TGF-ß are added. The next phase in the interaction in transmission of short-lived apoptosis-inducing factors between TGF-ß-pretreated normal cells and transformed cells. This process requires several hours or days, depending on the relative sensitivity of the transformed. It is followed by apoptosis of transformed cells. Initial experiments have already shown that reactive oxygen species have a role during elimination of transformed cells (Jürgensmeier et al., 1994b; Schaefer et al., 1995). Separation of the elimination system into three distinct phases, as well as finding an easy way to handle transformed and normal cells separately, has enabled us to define the exact point of interference of antioxidants (Langer et al., 1995). Antioxidants interfere with TGF-ß-dependent induction of apoptosis-inducing signal production in normal cells and with the TGF-ß-independent signal transfer between normal and transformed cells. The use of antioxidants that can reach intracellular locations or those that are restricted to the outside of cells has demonstrated that antioxidants interfere with an intracellular event during the induction process in normal cells and with extracellular events during signal transfer. It is

Fig. 5. Clonal analysis of normal cell apoptosis-inducing potential. Series A and B show the rational of the experiment. Normal C3H10T1/2 cells were seeded at low density (A1/B1) and allowed to form clones consisting of 25-50 cells per clone (A2/B2). Then transformed cells were added (marked in black in A3/B3). Cocultures under A remained free of exogenous TGF-ß, whereas TGF-ß was added to assays shown under B. After cocultivation for 4 days, assays were fixed and stained with Giemsa's solution. In TGF-ß-containing cocultures, all clones of normal cells were free of transformed cells, showing the picture as demonstrated under E. In the absence of exogenous TGF-ß, most of the clones of normal cells were covered with transformed cells (as shown for one clone under D). The experiment presented under C was performed according to this protocol. increasing concentrations of TGF-ß were used, as indicated. After four days of coculture clones without apoptotic transformed cells (open bars), clones with incomplete elimination of transformed cells (hatched bars) and clones with complete elimination of transformed cell (closed bars) were quantified. In the absence of exogenous TGF-ß, most clones of normal cells do not induce apoptosis of transformed cells, except for some. The heterogeneity of normal cells with respect to sensitivity of inducing TGF-ß becomes more prominent when suboptimal concentrations of TGF-ß are applied (e.g. 0.6 ng/nl). At optimal concentrations of TGF-ß, all clones of normal cells induce apoptosis of transformed cells. Data have been taken from Picht et al. 1995.

conceivable that these extracellular events are located directly at the cell membrane. Interference of antioxidants with signalling leading to apoptosis is in line with the suggestion that apoptosis is mediated by reactive oxygen species (Hockenbery et al., 1993; Kane et al., 1993). Our data implicates the function of oxygen-dependent steps at the beginning of the biochemical events leading to apoptosis in transformed cells.



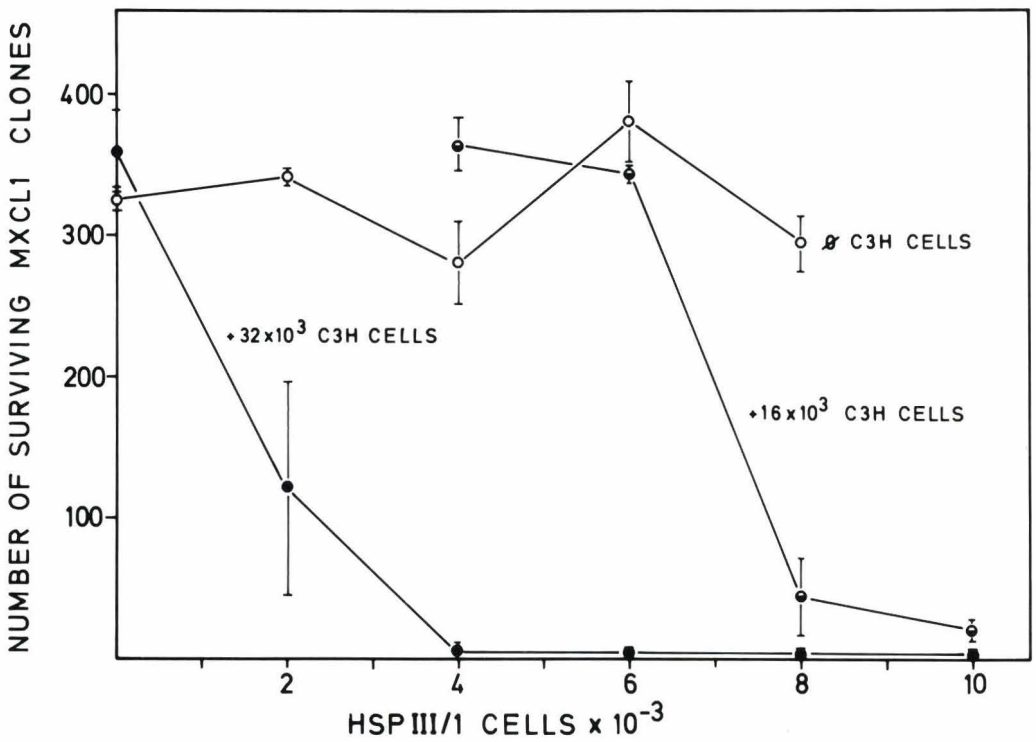


Fig. 6. Transformed cells trigger induction of their own apoptosis. The upper part of the graph shows the rationale of our experiment. Excess normal cells, sensitive to G 418 (N (G^s)) and small numbers of G 418-resistant transformed «indicator» cells (T (G^r)), indicated as open triangles, are cocultivated in the absence or presence of TGF-ß for 4-5 days. Then G 418 is added. Transformed indicator cells survive and form clones when TGF-ß was absent. In assays with TGF-ß during coculture, these cells have been eliminated; no surviving clones are found. Addition of large numbers of G 418-sensitive transformed cells (T(G^s), closed triangles)to cocultures of normal cells and indicator cells has the same effect as addition of TGF-ß: the indicator cells are eliminated. The lower part of the graph presents measured data from such an assay. 16000 or 32000 normal C3H10T1/2 cells were cocultivated with the indicated numbers of G 418-sensitive transformed HSP III/1 cells (a rat cell line transformed by HSV-1 as described by Bauer et al., 1992) and 500 transformed, G418-resistant indicator cells (MxCl1). After 5 days of coculture, the number of surviving clones of indicator cells was determined by G418 selection. Note that cocultivation of transformed cells without normal cells and no effect on the indicator cells. These data demonstrate that transformed cells can trigger induction of apoptosis. Parallel controls show that anti-TGF-ß inhibits triggering of induction of apoptosis, pointing to a role for TGF-ß-released from transformed cell themselves. These data have been taken from Hackenjos et al., 1995.

Interference with the TGF-ß induced process in normal cells points to a specific role of reactive oxygen species in the TGF-ß signalling pathway. This finding adds essential information to the understanding of TGF-ß action beyond activation of its receptors and is in line with the findings in other cellular systems (Das and Franburg, 1991; Das et al., 1992; Datta et al., 1992; Stenius, 1993). It confirms the concept of physiological functions fulfilled by reactive oxygen species as second messengers (Murell et al., 1990; Schreck et al., 1991; Schreck and Baeuerle, 1991; Amstad et al., 1992; Baeuerle and Henkel, 1994).

Due to its shortlived nature, the apoptosis-inducing signal itself remains undefined at present. Also, the basis for the specificity of its action - transformed cells versus normal cells - remains obscure.

IV. Induction of transformed cell apoptosis: indications for its efficacy in vitro

There are several lines of evidence that indicate that elimination of transformed cells by neighbouring normal cells acts as an efficient control of carcinogenesis in vitro. In addition, a critical review of previous work by other groups, which has been discussed so far on the basis of inhibition of proliferation, allows to speculate that induction of cell death may represent a possible explanation for the effects observed, as well. The following chapter summarizes several findings that are in line with elimination of transformed cells as a regulatory effect during carcinogenesis in vitro.

The transforming potential of HSV is a matter of controversial discussion. In vitro transformation assays

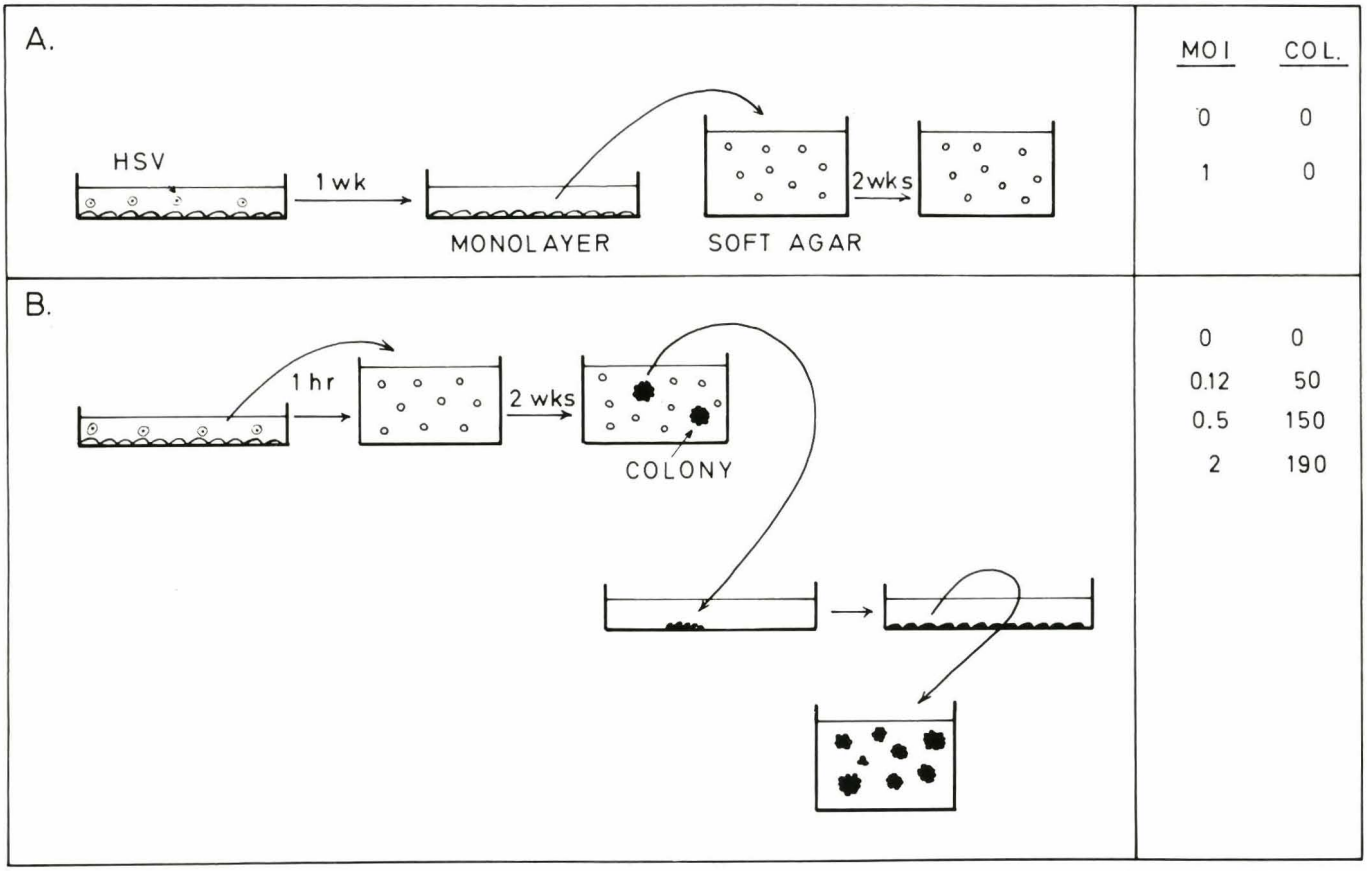


Fig. 7. Transformation of rat fibroblasts by HSV-1 in vitro is controlled by elimination of transformed cells. **A.** A monolayer of rat fibroblasts was infected with HSV-1 (multiplicity of infection (MOI= 1), i.e. one virus per cell) or remained uninfected (MOI= 0). After one week culture in monolayer, cells were trypsinized and seeded in soft agar, to score for transformed cells that form colonies. If the experiment was performed according to this protocol, no colonies were detectable. **B.** Monolayers of cells were infected with HSV (increasing MOIs) or remained uninfected. Already one hour after infection, cells were trypsinized and seeded in soft agar. Low cell density in soft agar prevents elimination of the arising transformed cells. Thus, stable transformation induced by HSV was demonstrated as colony formation that increased with the original concentration of HSV. Culture of isolated individual transformed colonies and retesting in soft agar showed, that they maintained the transformed state, i.e. they formed many colonies. These experiments were performed with a temperature sensitive mutant of HSV which allowed expression of immediate early and early genes at 39 ºC, but did not allow replication of viral DNA, production of virion particles and thus prevented lysis of cells. Note that the transformed state of daughter cells was maintained in the absence of viral DNA, thus directly proving the «hit-and-run» hypothesis for the transforming activity of HSV (Skinner, 1976; Galloway and McDougall, 1983). This experiment demonstrates that elimination of transformed cells by their neighbouring normal cells may efficiently suppress measurable transformation. Quantitative transformation studies therefore have to minimize elimination. Data have been taken from Bauer et al. 1992.

with this virus have only yielded marginal transformation frequencies, thus hampering the generic analysis of transformation by HSV. We used temperature-sensitive HSV mutants which, at the nonpermissive temperature allow expression of immediate early and early genes, but do not permit viral DNA synthesis (Bauer et al., 1992). This strategy prevented virus synthesis and lysis of infected cells. Infection of monolayer cultures with these mutant viruses followed by test for transformed cells one week later in soft agar did not lead to a detectable number of transformed colonies (Fig. 7). However, when the cells were separated immediately after adsorption and penetration of the virus, a significant number of transformed clones arose in soft agar. The efficiency of transformation was dependent on the initial multiplicity of infection. These results are best explained by the efficient killing of HSV-transformed cells in monolayer culture. Immediate separation of infected cells and sparse seeding in soft agar reduces the cell density to a level that is no longer sufficient for induction of apopotis. Triggering of induction of apoptosis by HSV-transformed cells was experimentally confirmed. In the meantime, this transformation system has enabled us to define one of the immediate early genes of HSV as the responsible gene for induction of transformation (Descalzo et al., in preparation). Extrapolation of these findings to the in vivo situation predicts that transformation by HSV is most likely abortive, unless mechanisms that protect transformed cells are acting in parallel.

In addition to the classical reversible induction of the transformed phenotype (in the presence of EGF) in rat fibroblasts, TGF-ß applied alone can also induce stable transformation in a small, but significant number of cells (Vossbeck et al., 1995). However, stable transformation of rat fibroblasts by TGF-ß can only be measured when cells are seeded at low density in soft agar, but not when they are treated in monolayer cultures. Efficiency of transformation in soft agar seems to depend on the abrogation of eliminative potential due to low total cell density. In monolayer, transformation and elimination occur in parallel. The net balance does not show transformed cells, as the eliminative process is dominant. This experimental system has provided interesting insights into the involvement of specific subpopulations of cells in the transformation process (Dichgans et al., 1995). TGF-ß treatment of a monolayer leads to transformation and subsequent elimination of this transformable subpopulation. Thus the culture is depleted of transformable cells and subsequent TGF-ß treatment in soft agar does not lead to colony formation.

As inhibition of transformed cell proliferation by surrounding normal cells has been the dominant model throughout the literature until recently, data that would have waranted interpretation as specific killing of transformed cells were usually interpreted by growth inhibitory mechanism. For example the findings by Silingardi et al. (1994) are parallel to our findings. It would appear to us that their data would be better explained by elimination of transformed cells than by inhibition of proliferation. The authors report a massive decrease in colony numbers, which can hardly be explained by inhibition of proliferation alone. Their statement that 60% of the cells are killed is in favour of our model.

The findings by Martin et al. (1991) might also imply elimination of transformed cells by neighbouring normal cells, in addition to the observed inhibition of proliferation. The authors state that the effect was reversible, which would be contradictory to the assumption of elimination. However, the test for reversibility was performed after only three days of coculture, a point of time at which, according to the kinetic data obtained in our system, elimination would not yet have been fully effective.

Herschmann and Brankow (1987) state that the constraint of growth control of transformed cells in coculture with normal cells was not effective when TPA was applied late. This finding may indicate elimination of transformed cells.

The necessity to kill surrounding normal cells for the demonstration of ras-induced transformation represents another example for the potential killing of transformed cells by normal cells (Spandidos and Wilkie, 1984; Land et al., 1986) and for the requirement for abrogation of elimination conditions for an optimal measurement of true transformation frequencies.

The biological parameters that have been found to be inhibitory to measurable transformation or focus reconstitution in many papers (Reznikoff et al., 1973; Bertram, 1977; Haber et al., 1977; Mordan et al., 1983; Schechtman et al., 1987) are to a large extent congruent with parameters necessary for induction of elimination of transformed cells by normal cells. High cell density, a common feature in all these systems, thereby must not necessarily indicate the involvement of direct cell-to-cell contacts, but also may reflect the need for a high number of normal effector cells for the process of elimination. The role of serum in inhibition, and the differences observed for different lots of serum may reflect differences in the content of active TGF-ß. Also, the increase in efficiency of inhibition dependent on the passage number, as stated by Frazelle et al. (1983) is in accordance with the findings for elimination of transformed cells in our system. It is not unlikely that one of the reasons for elimination observed in transformation systems is induction of elimination, triggered by the TGF-ß released by transformed cells. Production of TGF-ß seems to be a common feature of transformed cells (Moses et al., 1981; Roberts et al., 1982; Anzano et al., 1983; Nickel et al., 1983; Tucker et al., 1983; Massagué, 1984; Derynck et al., 1985, 1987; Goustin et al., 1986; Keski-Oja et al., 1987). This effect may act in concert with inhibition of proliferation or induction of differentiation. Vice versa, reduction of the pool size of transformed cells by inhibition might be an excellent prerequisite to allow efficient elimination of transformed cells and thus to establish tight control.

Initiation promotion experiments in vitro require

initial low cell density. This necessity has been interpreted as the need of sufficient rounds of replication of treated cells. We performed model experiments that used higher initial cell densities, but also allowed more rounds of replication, by reseeding the cells. These experiments showed that the efficiency of measurable transformation was heavily reduced at high initial cell density, even if the same number of cell cycles were allowed as under standard conditions. This data also points to the efficient control of carcinogenesis by normal cells at optimal density.

Elimination of spontaneously transformed cells in monolayer culture may be one of the central effects in the system described by Rubin et al. (Rubin and Xu, 1989; Rubin et al., 1990; Farber and Rubin, 1991). The inhibitory effect of high serum concentrations is also in accordance with this assumption. Work in progress in our laboratory shows that NIH 3T3 cells show a significantly high spontaneous transformation rate which is efficiently controlled by elimination. This interplay leads to a marginal net balance in favour of transformed cells arising spontaneously. It is obvious that any factor that interferes with this delicate balance by inhibiting elimination will initially appear like a transforming agent. This concept has to be kept in mind when results from cell cultures with potentially high or unknown spontaneous rates are interpreted in terms of induction of transformation. It may require a distinct approach to distinguish true induction of transformation from protection of spontaneously transformed cells from elimination by the normal cells of the culture.

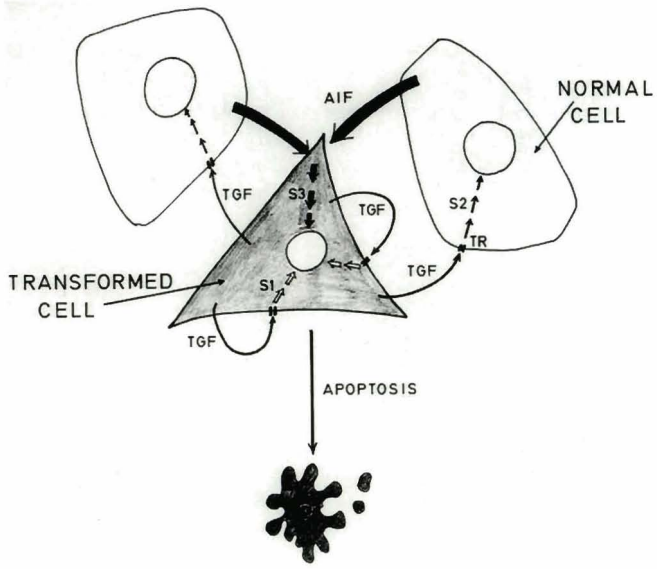


Fig. 8. Elimination of transformed cells by normal cells through induction of apoptosis. Summary of established data. The figure summarizes the established data on the intercellular control step described by us. Transformed cells release active TGF-ß and maintain their transformed state by an autocrine loop (Wehrle et al., 1994). TGF-ß released from transformed cells (Hackenjos et al., 1995) induces production of short-lived apoptosis-inducing factor (AIF) in normal cells (Jürgensmeier et al., 1994b). This factor acts on transformed cells, leading to apoptosis (Jürgensmeier et al., 1994a). All cells within the population of normal cells are able to produce apoptosis-inducing factors (Picht et al., 1995). Both the TGF-ß-induced signal chain in normal cells and the interaction of apoptosis-inducing factors with transformed cells are dependent on the specific action of reactive oxygen species (Langer et al., 1995). Presence of antioxidants therefore interferes with the elimination of transformed cells (Jürgensmeier et al., 1994b; Schaefer et al., 1995). Cells transformed in vitro are generally sensitive to induction of apoptosis, independent of the agent that originally caused their transformation (Jürgensmeier et al., 1994a). Cells transformed by bovine papillomaviruses represent the only exception found so far (Melchinger et al., unpublished). Ex vivo tumor lines are generally resistant against induction of elimination (Schaefer, unpublished; Engel, unpublished; Brauns, unpublished), indicating that tumor progression in vivo may require acquisition of resistance against this control mechanism. TGF: TGF-ß; TR: TGF-ß receptor; AIF: apoptosis inducing factor; S1: TGF-ß-induced signal pathway leading to the maintenance of the transformed state; S2: TGF-ß-induced signal pathway leading to the production of AIF; S3: signal pathway induced by AIF and leading to apoptosis. The graph and its legend have been taken from Bauer, 1995.

The ability of transformed cell to form foci in reconstitution systems is one of the traits of transformed cells. In the light of our findings, low efficiency of focus formation by chemically transformed cells (Smith et al., 1993) may also be due to an efficient induction of apoptosis by the transformed cells themselves. Cells with low efficiency of focus forming ability may be those that produce the most TGF-ß and thus trigger induction of their own apoptosis most efficiently. It is obvious, that the density of normal cells used as well as their passage number will influence the efficiency of focus formation. Based on ongoing work in our laboratory, the use of gamma-irradiated normal cells seems to be a more precise means of measuring focus forming ability, as these cells are inhibited to perform induction of apoptosis (Bassler et al., in preparation). In the light of these data it is not clear whether the inability of cells transfected with an active TGF-α gene to form foci (Finzi et al., 1987) indeed reflects the inability of TGF-α to induce or maintain stable transformation, or whether detection of potentially transformed cells has been hampered due to efficient elimination of arising transformed cells.

V. Predictions and hypothesis based on the novel concept of transformed cell elimination by normal cells

The established data on the interaction of transformed and normal fibroblasts are summarized in Fig. 8. Transformed cells release active TGF-ß, which is required for the maintenance of their transformed state (Wehrle et al., 1994). Release of TGF-ß is a common feature of transformed cells (Moses et al., 1981; Roberts et al., 1982; Anzano et al., 1983; Nickel et al., 1983; Tucker et al., 1983; Massagué, 1984; Derynck et al., 1985, 1987; Goustin et al., 1986; Keski-Oja et al., 1987), thus the mechanism described by us may be significant for cells transformed by different transformation principles. In vivo, the concentration of TGF-ß established in the intercellular space by transformed cells is probably sufficient for induction of a process in

normal cells that leads to the production of apoptosis-inducing factors. The TGF-ß-dependent signal pathway involved in this process seems to depend on reactive oxygen species (Jürgensmeier et al., 1994b; Schaefer et al., 1995; Langer et al., 1995). The apoptosis-inducing signal acts specifically on transformed cells in a ROS-dependent pathway. Sensitivity to induction of apoptosis seems to be a general feature of transformed cells, independent of the principle that cause their transformation (Jürgensmeier et al., 1994a).

If this mechanism acts in vivo, in addition to acquisition of immortalization, activation of oncogenes and loss of tumor suppressor genes, cells need mechanisms to overcome TGF-ß mediated induction of apoptosis by neighbouring cells in order to enable tumor formation. Induction of apoptosis in transformed cells might reflect an early and dominant anticarcinogenic effect: a potential novel control point of carcinogenesis. Similarly to the cellular immune system it acts by destruction of tumor cells. However, its action might be immediately after establishment of the transformed phenotype.

Efficient tumor formation therefore should require mechanisms to overcome this control (Fig. 9). Several mechanisms are conceivable (Bauer, 1995). Chemicals interfering with elimination might represent efficient

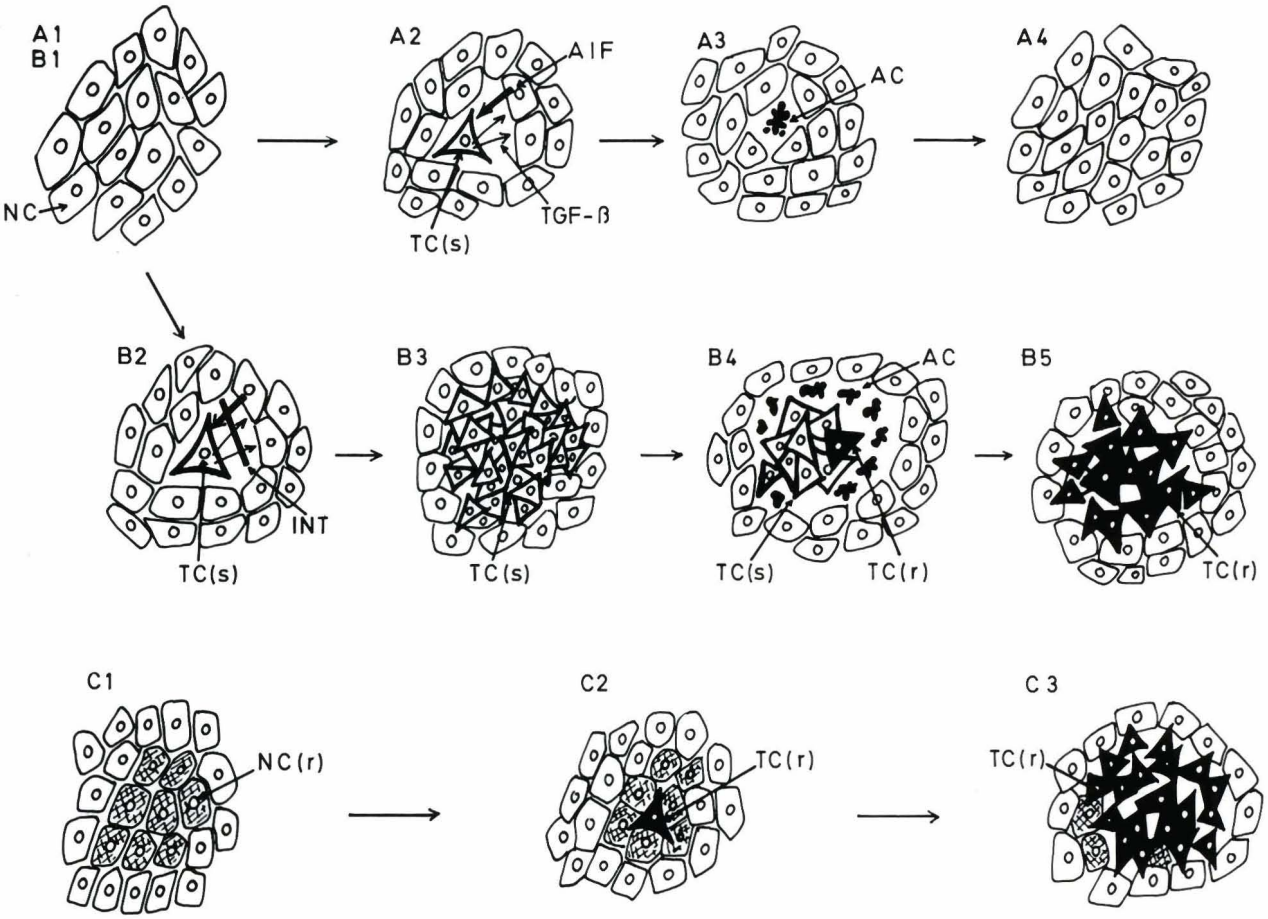


Fig. 9. Tumor progression requires acquisition of resistance against TGF-ß-induced elimination of transformed cells by normal cells. This figure summarizes the predictions from our data obtained in vitro for the situation in vivo. **A.** Transformation events within a population of normal cells (A1/B1) lead to transformed cells that are potentially sensitive against induction of apoptosis by adjoining normal cells (A2). Arising transformed cells release TGF-ß and thus induce normal cells to produce an apoptosis-inducing factor (A2) that eliminates the transformed cells (A3, A4). As induction of elimination is a fast process, acquisition of resistance after transformation is unlikely to be effective. The mechanism described here might represent an effective control of carcinogenesis. **B.** Presence of substances that interfere with the elimination process (like antioxidants and radical scavengers) (B2) allows elimination-sensitive transformed cells to survive and to proliferate (B3). When the interfering substance is no longer available (B4), the cells at the outer surface of the micro-tumor are subject to induction of apoptosis by adjacent normal cells, whereas the cells within the tumor continue to proliferate. This situation might allow selection of resistant transformed cells that finally form a tumor, consisting of resistant cells (B5). **C.** If a tissue contains cells that express a mechanism that renders them elimination-resistant after subsequent transformation (C1), transformation of any of these cells will lead to elimination-resistant transformed cells immediately (C2). These cells can form a tumor without being subject to the above described intercellular control mechanism (C3). NC: normal cell; TC(s): transformed cell, sensitive for induction of apoptosis; TC(r): transformed cell, resistant to induction of apoptosis; AIF: apoptosis-inducing factor; INT: substances interfering with induction of elimination (e.g. antioxidants or radical scavengers); NC(r): normal cell, which after transformation, gives rise to transformed cells resistant to induction of elimination. This figure and its legend have been taken from Bauer, 1995.

means to protect transformed cells and to stabilize their population. Reconstitution experiments have shown that antioxidants like catechol or hydroquinone can protect transformed cells from basal elimination and from elimination induced by exogenous TGF-ß (Schaefer et al., 1995). This system might represent a model for chemicals acting in a pro-carcinogenic way without being involved in the transformation process directly. Protection of transformed cells might result in a growing tumor. In the absence of the action of further protecting chemicals, the cells inside the tumor are inaccessible for apoptosis-inducing signals from neighbouring normal cells. Only the cells in the area between the border of the tumor and normal cells are subject to induction of apoptosis. It is conceivable that under these conditions selection of resistant tumor cells might occur. Resistant cells then might grow out efficiently.

Resistance of transformed cells would represent the simplest way to overcome control by elimination. As induction of apoptosis is a fast acting process, it is unlikely that cells could become resistant fast enough to escape elimination after transformation. Biochemical mechanisms that lead to potential resistance of cells prior to transformation, however, might represent a high potential of protection of cells transformed by later events. The bcl-2 protein represents an interesting candidate for such a function. bcl-2 has been shown to interfere with the process of apoptosis (Hockenbery et al., 1990, 1993; Kane et al., 1993; Reed, 1994). Ongoing experiments in our laboratory show that bcl-2 can also protect transformed cells from induction of apoptosis by TGF-ß-treated neighbouring cells (Jürgensmeier and Bauer, submitted). If the bcl-2 gene is activated prior to transformation, it may allow escape of transformed cells from the intercellular control step described here. This hypothesis is in perfect accordance with recent findings on the activation of bcl-2 as an early step in carcinogenesis in vivo (Bronner et al., 1995). As certain genes of DNA viruses act analogously to bcl-2-like adenovirus E1B (Rao et al., 1992), EBV BHRF1 (Henderson et al., 1993) or activate bcl-2, like LMP1 of EBV (Henderson et al., 1991), protection of transformed cells by these genes may be an hitherto unrecognized crucial event during carcinogenesis. It allows to define a «protector function» of DNA tumor viruses and related cellular genes which cooperates with classical transforming activities.

In line with this hypothesis is our finding that papillomavirus-transformed cells represent the only exception of in vitro transformed cells with respect to sensitivity to induction of apoptosis. Papilloma-virus transformed cells are resistant and thus it seems that papilloma viruses not only are involved in the induction of transformation but also in the protection of arising transformed cells from negative effects of neighbouring cells (Melchinger et al., in preparation). A tissue infected with papilloma viruses therefore might represent an ideal substrate for the tumorigenic action of other carcinogens, as it protects the arising transformed cells from cell death. Work on the interaction of bovine papillomatosis and the intake of carcinogens from bracken fern is in perfect agreement with the predictions from our model. In this system, uptake of chemical carcinogens only causes tumor formation on the basis of a pre-existing papilloma virus infection (Gaukroger et al., 1993; Campo et al., 1994). These findings are paralleled by results obtained in vitro (Pennie and Campo, 1992).

The sensitivity of in vitro transformed cell lines and the resistance of ex vivo tumor lines against induction of apoptosis by neighbouring cells meets the requirements of this concept. Resistance might be based on the inability to recognize apoptosis-inducing signals or on effective interference with the signalling pathway induced by such signals. The metabolism of reactive oxygen and of intracellular antioxidants may be a clue to these problems.

The inefficiency of normal cells to produce apoptosis-inducing signals might equally favor the development of tumors.

Further studies along these lines may lead us to new insights into the mechanism of tumor progression. The study of the mechanisms of induction of apoptosis in transformed cells may reveal specific pathways of transformed cells. The elucidation of the mechanism of resistance of tumor cells hopefully also inherits potential therapeutic and prognostic significance.

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