**ras oncogene expression determines sensitivity for intercellular induction of apoptosis**

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**Fibroblasts carrying an inducible ras oncogene acquire the transformed phenotype after oncogene induction.** As a consequence, the transformed cells become sensitive to intercellular induction of apoptosis, a novel regulatory process directed by non-transformed fibroblasts against their transformed descendants. The causal relationship between oncogene expression and sensitivity to intercellular induction of apoptosis is based on extracellular superoxide anion production by oncogene-expressing cells. Superoxide anions (after dismutation to hydrogen peroxide) thereby foster HOCl synthesis and at the same time direct the selectivity of apoptosis induction through hydroxyl generation from HOCl. In parallel, ras expression enhances the sensitivity of fibroblasts for apoptosis-inducing stimuli like cycloheximide, ceramide and mitomycin C. This sensitization seems to be based on a decreased concentration of short lived endogenous apoptosis inhibitors. TGF-β, like ras induction, decreases the sensitivity to endogenous apoptosis inhibitors, but does not induce the transformed phenotype. Therefore, TGF-β treatment alone is not sufficient to render fibroblasts sensitive for intercellular induction of apoptosis, but TGF-β treatment in parallel with ras activation enhances intercellular induction of apoptosis. Our findings demonstrate that Ras-mediated superoxide anion production determines sensitivity to intercellular induction of apoptosis, whereas the parallel decrease in endogenous apoptosis inhibitors modulates the kinetics of apoptosis induction.

**Introduction**

Activating mutations within the N-ras, K-ras and H-ras genes frequently contribute to the development of human tumours of myeloid and epithelial origin (1). ras oncogenes can induce cellular transformation in various cell types in vivo and in vitro (2,3) and are frequently mutated in experimental tumours (4). The products of the ras genes belong to the superfamily of small GTP-binding proteins. These are key elements in signal transduction pathways which couple external growth factor stimuli with intracellular events resulting in differentiation or mitogenesis (for reviews see refs 5,6). ras expression activates rac and, as a consequence, a membrane-associated NADPH oxidase generates extracellular superoxide anions (7,8). These seem to be crucial for mitogenesis and maintenance of the transformed state of ras-expressing cells.

The influence of ras oncogene expression on cell survival rather than on its transformed state has been studied a lot less and has led to conflicting results. ras expression was shown to cause increased sensitivity to apoptosis induction by TNF (9–11). On the other hand, ras expression inhibits drug- and UV-induced apoptosis (12) and is functionally involved in FGF-dependent suppression of apoptosis (13).

Transformed fibroblasts are subject to control by non-transformed neighbouring cells through intercellular induction of apoptosis, a potential control mechanism during oncogenesis (14–21). TGF-β and FGF trigger non-transformed cells (effector cells) to induce apoptosis in transformed fibroblasts (target cells) in a process that does not depend on direct cell–cell contact between effector and target cells and which is mediated by reactive oxygen species (17–21). Intercellular signalling during apoptosis induction in transformed fibroblasts by TGF-β-treated non-transformed cells thereby seems to depend on superoxide anions produced by the target cells and a novel peroxidase released by non-transformed effector cells (17). This enzyme utilizes hydrogen peroxide (generated by spontaneous dismutation of superoxide anions) to synthesize hypochlorous acid. Hypochlorous acid is then converted to apoptosis-inducing hydroxyl radicals through interaction with superoxide anions according to the formula HOCl + O2 → OH + Cl + O2 (17,18). In addition, nitric oxide released by effector cells seems to interact with target cell-derived superoxide anions and forms apoptosis-inducing peroxynitrite (17). Intercellular induction of apoptosis does not depend on p53 (22). Certain anti-apoptotic genetic systems like bcl-2 (23) or bovine papilloma viruses (24) confer resistance to intercellular induction of apoptosis and thus may result in protection of transformed cells from intercellular control. One of the crucial questions in this system is whether a causal relationship exists between establishment of the transformed state, extracellular superoxide anion generation and sensitivity to intercellular induction. The availability of 208 F fibroblasts with an inducible ras oncogene allowed us to address this question directly. In addition, it was interesting to find out whether ras expression would influence the response of the cells to other apoptosis inducers.

**Materials and methods**

**Materials**

TGF-β1 has been purified from human platelets as recently described (14). EGF was obtained from Sigma (Deisenhofen, Germany). Factors were dissolved in medium containing 5% fetal calf serum and were stored as aliquots at −20°C. Superoxide dismutase (SOD) (from bovine erythrocytes; Sigma) stock solutions (30 000 U/ml in phosphate-buffered saline) were kept at −20°C and only used once per aliquot. Taurine (Sigma) was kept as a stock solution of 500 mM in medium at −20°C. The solution had been passed through a sterile filter. Taurine represents a specific scavenger of HOCl (25). Catalase from Aspergillus niger (77 U/μl) and Terephthalate (sodium salt) were obtained from Sigma. The latter was kept as a stock solution of 40 mM
in water at -20°C. Terephthalate represents a specific scavenger of hydroxyl radicals (26). The NO donor sodium nitroprusside (SNP) was obtained from Sigma and was kept as a stock solution of 200 mM in medium at -20°C. The cell-permeable ceramide C2 was obtained from Sigma and was kept as a stock solution of 40 mM in dimethyl sulfoxide.

Cell culture
Cells were kept in Eagle's minimal essential medium (MEM) containing 5% fetal calf serum that had been heated for 30 min at 56°C prior to use. Medium was supplemented with penicillin (40 U/ml), streptomycin (50 µg/ml), neomycin (10 µg/ml), moronal (10 U/ml) and glutamine (280 µg/ml). Cell culture was performed in plastic tissue culture flasks. Cells were passaged once or twice weekly. For maintenance of plasmid-coded G418 resistance, cell lines were kept in the presence of 0.5 mg/ml G418. Untransformed mouse C3H 10 T1/2 fibroblasts were obtained from U. Rapp (University of Würzburg, Germany). The cells do not form colonies in soft agar and exhibit a very low rate of spontaneous transformation.

208 F cells with an inducible ras oncogene
The establishment of 208 F cells with inducible H-ras will be described elsewhere (Scharm et al., in preparation). Briefly, immortalized 208 F rat fibroblasts were transected with the H-ras oncogene under control of the SV40 promotor and the lac operator sequence, the lac repressor gene and pSV2neo. Three distinct inducible clones, named IR1, IR2 and IR8, were used in this study. For induction of ras expression, the cells received 20 mM isopropyl-β-D-thiogalactoside (IPTG). IPTG was purchased from Sigma (Deisenhofen, Germany).

Test for colony formation in soft agar
The tests were performed as recently described (27).

Determination of apoptosis
Determination and quantification of apoptosis were based on the classical morphological criteria membrane blebbing, nuclear condensation and nuclear fragmentation. These were determined using inverted phase contrast microscopy, as recently described (14). The percentage of apoptotic cells was determined from at least 200 cells categorized per assay. Apoptotic cells were either attached or rounded and showed (i) membrane blebbing or (ii) membrane blebbing and nuclear condensation/fragmentation or (iii) nuclear fragmentation/condensation without blebbing (these cells seem to represent later stages of apoptosis where the blebs have already been lost). Care was taken to differentiate apoptotic cells from non-apoptotic rounded cells with intact nuclei, reflecting mitotic stages.

All quantitative data in this paper were derived using this method. In parallel, control assays ensured that apoptotic cells characterized by morphological criteria as described above showed nuclear condensation/fragmentation determined by staining with bis-benzimide and a positive TUNEL reaction (28), indicative of free 3'-hydroxyl groups on the DNA, one of the hallmarks of apoptotic cells.

For bis-benzimide staining cells were rinsed with phosphate-buffered saline and incubated with 1 µg/ml bis-benzimidine (Hoechst 33258 fluorescein) (0.9 mM acetic acid, 1 mM potassium acetate in methanol) for 30 min. After washing with aqua bidest, cells were embedded in MclIvane/glycerol (McIvane's citrate–phosphate buffer, 50 mM potassium dihydrogen phosphate, 50 mM citric acid, pH 5.5/glycerol 1:1). Cells were examined with a fluorescence microscope, using excitation at 395–440 nm.

DNA strand breaks (free 3'-hydroxy groups) were detected by the TUNEL reaction (28), using a commercially available detection kit (Boehringer, Mannheim, Germany). It is based on the incorporation of fluorescein-labelled dUTP by terminal deoxynucleotidyl transferase. Representative photographs of apoptotic transformed and non-transformed fibroblasts stained with bis-benzimide and the TUNEL reaction have recently been published (15,22), therefore no pictures are shown here.

Test for induction of apoptosis in transformed target cells by TGF-β1-treated non-transformed effector cells (intercellular induction of apoptosis)
For co-cultivation of target and effector cells without cell–cell contact, cell culture clusters with tissue culture inserts were used (Falcon, obtained from Becton Dickinson, Heidelberg, Germany), distance between cell layers ~2 mm. Effector cells: Non-transformed 208 F effector cells were seeded into the inserts (4×10⁴ cells/insert). After the cells attached, they were treated with 20 ng/ml TGF-β2 for 2 days (37°C, 5% CO₂). The medium was then removed, the inserts were washed with medium and placed above the target cells in 6-well plates.

Target cells: IR-1 cells were either pretreated with 20 mM IPTG for 2 days to induce the transformed phenotype or remained uninduced in control assays. Cells were trypsinized and seeded in clumps of high local density. Aliquots of 2500 cells in 7 µl of medium were placed in Costar 6-well tissue culture clusters (4 spots/assay). After the cells had attached (<3 h at 37°C), 2 ml of medium were added. IPTG treatment was continued in IPTG-pre-treated cultures, in order to maintain the transformed phenotype. Tissue culture inserts containing the TGF-β1-pre-treated non-transformed effector cells were placed above the target cells. After the indicated time of co-culture, the target cells were checked for apoptotic cells (membrane blebbing, chromatin condensation/fragmentation) using phase contrast microscopy as described (14).

Seeding the target cells at relatively low number (4×10⁴/2500 cells/assay) but at high local density (200 cells/mm²) is important for efficient intercellular induction of apoptosis. The low total cell number prevents depletion of the medium. The high local density of the cells ensures efficient spontaneous dismutation of transformed cell-derived extracellular superoxide anions to hydrogen peroxide. The hydrogen peroxide concentration is rate limiting for the subsequent peroxidase reaction, one of the central steps in apoptotic signalling during intercellular induction of apoptosis (17,18). As outlined in the Introduction, peroxidase generates hypochlorous acid, which then interacts with target cell-derived superoxide anions to generate apoptosis-inducing hydroxyl radicals.

Clonal analysis of intercellular induction of apoptosis
Samples of 80–100 clones of IPTG-treated or untreated IR-1 or IR-2 cells were grown in Costar 6-well tissue culture clusters in MEM containing 5% fetal calf serum and 20% conditioned medium. Addition of conditioned medium (medium from a semi-dense cell culture of IR-1 or IR-2 cells) is required for initial survival of the cells after seeding. After the clones had reached a size of ~50 cells/clone, they were labelled by scratching the tissue culture clusters from underneath with a needle. Medium was renewed (including IPTG where IPTG had been present before) and 6×10⁴ non-transformed effector cells were added. After these were attached, TGF-β1 (10 ng/ml) was added. After 3–5 days of co-culture, clones of transformed cells were scored for characteristics of apoptosis (membrane blebbing, chromatin condensation/fragmentation). Clones were categorized as exhibiting <10% apoptotic cells, between 10 and 50% apoptotic cells or >50% apoptotic cells.

Demonstration of extracellular superoxide anion production by transformed cells
The functional assay for extracellular superoxide anion generation is based on the induction of extracellular superoxide anions with nitric oxide (NO) generated by the NO donor SNP. Whereas NO has no direct apoptosis-inducing potential, peroxynitrite resulting from NO/superoxide anion interaction (in a diffusion-driven reaction) represents a highly efficient apoptosis inducer (17). The functional role of superoxide anions is determined by inhibition of apoptosis by the superoxide anion scavenging enzyme SOD. SOD-sensitive apoptosis induction in the presence of SNP indicates extracellular superoxide anion generation, as SOD cannot penetrate fibroblasts.

Results
In order to test for a correlation between ras oncogene induction and subsequent expression of the transformed phenotype, 208 F rat fibroblasts containing an IPTG-inducible ras oncogene expression plasmid (named IR cells) were cultivated in the presence or absence of the inducer. Culture was either performed clonally in monolayers or in soft agar. Induction of ras oncogene expression by addition of IPTG resulted in crisis-cross morphology in 99% of the clones in the case of IR-1 cells and in 98% of the clones in the case of IR-2 cells. IR-1 or IR-2 cells treated with IPTG for 2 days showed expression of Ras protein, detectable by immunoblot (data not shown). A crisis-cross morphology was present in 4.5% of IR-1 clones and 7.3% of IR-2 clones in the absence of the inducer, thus demonstrating a low degree of leakiness of this oncogene induction system. A crisis-cross morphology in monolayer culture was paralleled by the ability of induced cells to form colonies in soft agar. Whereas less than 10% of the untreated IR-1 or IR-2 cells showed more than three doublings in soft agar, 89% of the IPTG-treated IR-1 cells and 82% of the IPTG-treated IR-2 cells showed more than three doublings. Sixty-six per cent of IPTG-treated IR-1 cells and 55% of IPTG-treated IR-2 cells showed five or more doublings. These data show that efficient induction of the morphological features
of the transformed state had occurred after ras expression in most of the cells of the population.

Superoxide anion production has been shown to represent a distinct biochemical feature of fibroblasts with constitutive ras expression (8). To test for extracellular superoxide anion production by fibroblasts with induced ras expression, IR-1 cells pretreated with IPTG or not, as well as parental fibroblasts without the ras-containing plasmid, were treated with the NO donor SNP. This treatment allows very sensitive detection of superoxide anions, as superoxide anions react rapidly with NO (k = 6.8×10⁷ s⁻¹ M⁻¹) (29–36) and form the apoptosis inducer peroxynitrite (17; Heigold et al., submitted for publication). Inclusion of control assays with additional SOD thereby allows definition of the role of superoxide anions in this reaction and determination of their extracellular location, as SOD cannot penetrate cells. As can be seen in Table I, NO efficiently mediated apoptosis induction in ras-transformed cells. This reaction was completely inhibited by SOD, indicating the involvement of superoxide anions. Non-induced IR cells showed weak apoptosis induction by NO, confirming the low degree of leakiness of the ras induction system. Plasmid-free parental cells were unaffected by NO, indicating that non-transformed fibroblasts do not generate a substantial concentration of extracellular superoxide anions. These results indicate that induced ras expression in our system causes marked extracellular superoxide anion generation, which renders ras-expressing cells sensitive to NO-mediated apoptosis (based on peroxynitrite formation).

To test whether establishment of the transformed state by ras expression caused induction of sensitivity of the cells for intercellular induction of apoptosis, tissue culture inserts containing TGF-β-pre-treated non-transformed 208 F fibroblasts (effector cells) were placed above IR cells with or without induced ras expression (target cells). Parallel assays with induced and non-induced cells remained free of tissue culture inserts. As can be seen in Figure 1, intercellular induction of apoptosis occurred specifically and exclusively in ras-expressing cells in co-culture with TGF-β-pre-treated non-transformed effector cells. None of the other assay combinations showed apoptosis induction above background. This finding demonstrates that sensitivity to intercellular induction of apoptosis is linked to expression of the transformed phenotype through ras.

When ras-expressing clones of IR cells (rather than a disperse culture of cells as shown in Figure 1) were challenged with TGF-β-treated non-transformed effector cells the majority of clones became apoptotic (data not shown). This finding confirms the results shown in Figure 1 and demonstrates that ras-expressing clones are rather homogeneous with respect to sensitivity for intercellular induction of apoptosis.

To clarify whether superoxide anions specifically generated by IR cells with induced ras expression were crucial for their sensitivity to intercellular induction of apoptosis, assays with ras-expressing cells as targets and non-transformed TGF-β-pre-treated effector cells were treated with SOD (to test for involvement of superoxide anions), taurine (to scavenge HOCl possibly generated by peroxidase), terephthalate (to test for a potential role of hydroxyl radicals) and catalase (to test for a role of hydrogen peroxide). As can be seen in Figure 2, each of these additions caused a marked inhibition of apoptosis induction in ras-transformed cells by non-transformed effector cells, indicating that superoxide anions and their reaction products hydrogen peroxide, hypochlorous acid and hydroxyl radicals played a central role in sensitivity and efficiency of intercellular induction of apoptosis.

It was of interest to find out whether ras expression

Table I. Generation of extracellular superoxide anions by ras-expressing fibroblasts detected by SOD-sensitive NO-mediated apoptosis induction

<table>
<thead>
<tr>
<th>Cell line</th>
<th>SNP (mM)</th>
<th>SOD (U/ml)</th>
<th>Apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>208 F</td>
<td>0.25</td>
<td>3 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>208 F</td>
<td>2</td>
<td>2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>IR-1</td>
<td>2.06</td>
<td>5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>IR-1</td>
<td>2.25</td>
<td>23 ± 7</td>
<td></td>
</tr>
<tr>
<td>IR-1 + IPTG</td>
<td>0.25</td>
<td>8 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>IR-1 + IPTG</td>
<td>0.06</td>
<td>2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>IR-1 + IPTG</td>
<td>0.25</td>
<td>68 ± 15</td>
<td></td>
</tr>
<tr>
<td>IR-1 + IPTG</td>
<td>0.25</td>
<td>7 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

Non-transformed 208 F fibroblasts, IR-1 cells and IPTG-pretreated IR-1 cells (pretreated for 2 days with 20 mM IPTG, then IPTG treatment continued) were seeded in Costar 24-well tissue culture clusters (15,000 cells/assay). The NO donor SNP was added at the indicated concentrations. Where indicated, assays received 150 U/ml SOD. All assays were performed in duplicate. The percentages of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) were determined after 64 h using inverted phase contrast microscopy.

Fig. 1. ras activation causes transformation and sensitivity to intercellular induction of apoptosis. IR-1 cells were pretreated with 20 mM IPTG or remained untreated. After 2 days, the IPTG-treated cell population showed the transformed morphology. Cells were trypsinized and seeded in Costar 6-well tissue culture clusters at four distinct spots (2500 cells each, 200 cells/mm²). This high local cell density enhances spontaneous dismutation of cell-derived superoxide anions to hydrogen peroxide (17,51). IPTG treatment was continued in the case of IPTG-pretreated cells, in order to maintain expression of the transformed phenotype. Tissue culture inserts containing 40,000 208 F effector cells (pretreated with 20 ng/ml TGF-β for 2 days) were placed above the IR cells. Parallel assays remained free of tissue culture inserts. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined kinetically using inverted phase contrast microscopy. All assays were performed in duplicate.
induced apoptosis in Inhibition of macromolecular synthesis has been shown to involve ROS during intercellular induction of apoptosis in ras-transformed IR cells. The experiment was performed as described in Figure 4. In addition, assays received 150 U/ml SOD, 25 mM taurine, 200 µM terephthalate or 77 U/ml catalase, as indicated in the figure. SOD scavenges superoxide anions, taurine scavenges HOCl, terephthalate is a specific hydroxyl radical scavenger and catalase destroys hydrogen peroxide. The percentage of apoptotic cells (characterized by membrane blebbing and/or nuclear condensation/fragmentation) was determined after 3 days. All assays were performed in duplicate.

influenced the sensitivity for other apoptosis inducers as well. Inhibition of macromolecular synthesis has been shown to induce apoptosis in fibroblasts (37,38). These findings have been interpreted as being indicative of a constitutively expressed apoptosis machinery which is controlled by inhibitory proteins, operationally termed endogenous survival factors (37,38). Due to their short half-life, inhibition of their synthesis releases the apoptosis machinery from negative control. The time between addition of cycloheximide (CHX) and the onset of apoptosis allows an estimation of the relative intracellular concentration of endogenous apoptosis inhibitory proteins (38). In order to test for the sensitivity of ras-expressing and control cells for apoptosis induction after abrogation of protein synthesis, IR cells were treated with IPTG for varying times or remained uninduced and then CHX was added. As shown in Figure 3, abrogation of protein synthesis caused a slow induction of apoptosis, starting ~6 h after addition of CHX, in control cells but not those treated with IPTG. In contrast, ras-expressing cells showed a rapid increase in apoptosis immediately after addition of CHX. The rate of CHX-mediated apoptosis was dependent on the length of pretreatment with IPTG. ras expression in the absence of CHX caused no apoptosis induction (Control). These findings show that ras-expressing fibroblasts are more sensitive for apoptosis induction through inhibition of protein synthesis than control cells. ras expression therefore seems to have decreased the concentration of endogenous apoptosis inhibitory proteins (endogenous survival factors). TGF-β treatment exhibited a similar effect on endogenous apoptosis inhibitory proteins as ras treatment (Figure 4), but was not sufficient to induce the transformed phenotype (data not shown). ras expression together with TGF-β treatment showed an additive effect on the down-modulation of apoptosis inhibitory proteins. These results indicate that TGF-β and ras expression have analogous (and additive) effects on the cellular apoptosis control system. In order to evaluate whether a decrease in endogenous apoptosis inhibitory proteins was sufficient to render cells sensitive for intercellular induction of apoptosis, IR cells with or without IPTG treatment for induction of ras expression were treated with TGF-β in parallel (or not). Then the effects on the transformed phenotype and sensitivity for intercellular induction of apoptosis were monitored. Cells induced with IPTG showed the transformed phenotype, independent of TGF-β treatment. Non-induced cells did not show the transformed phenotype. Treatment of non-induced cells with TGF-β was not sufficient to establish the transformed phenotype (data not shown). When challenged with TGF-β-pretreated effector cells, only cells expressing the transformed phenotype through ras induction showed sensitivity for intercellular induction of apoptosis (Table II). Non-transformed cells were insensitive for intercellular induction of apoptosis, independent of TGF-β pretreatment. TGF-β pretreatment of ras-expressing transformed cells caused an enhancement of apoptosis induction. These findings confirm that the transformed phenotype determines sensitivity for intercellular induction of apoptosis. A decrease in the concentration of endogenous apoptosis inhibitory proteins is not sufficient to induce sensitivity, but the survival factor concentration modulates the apoptosis-inducing effect on transformed cells that are already sensitive, as seen in the case of ras-expressing cells that had been treated with TGF-β in addition.

Mitomycin C is a classical inducer of p53-mediated apoptosis (39). To test ras-expressing and control cells for their sensitivity to mitomycin-induced apoptosis, IR cells were pretreated with IPTG or not, followed by mitomycin C treatment. Parallel assays did not receive mitomycin C. As shown in Figure 5, apoptosis induction by mitomycin in ras-expressing cells
occurred much faster than in control cells without ras expression. Therefore, apoptosis induction by mitomycin C per se does not depend on the transformed state, but ras expression enhances apoptosis induction by mitomycin C. Similarly, addition of the cell-permeable ceramide C2 induced apoptosis in non-transformed as well as in transformed fibroblasts, with an increased sensitivity of ras-expressing fibroblasts (Figure 6).

Discussion

Induction of ras expression in rat fibroblasts causes a rapid appearance of the morphological features of the transformed phenotype, characterized by a criss-cross morphology of the cells and their ability to grow anchorage independently. Anchorage-independent growth in soft agar represents a rather strict correlate of the transformed state and is indicative of the ability of cells to form tumors in nude mice (40). Clonal analysis of transformation parameters demonstrated that the induced cell population was rather homogeneous with respect to the expression of transformed features and that the non-induced controls showed only a minor degree of leakiness. This system therefore allows the study of biochemical and biological consequences caused by ras expression. As expression of transformed features depended strictly on addition of the specific inducer IPTG and as Ras protein was detectable in IPTG-treated cells, but not in untreated cells, the involvement of ras in the mechanisms studied is proved. 

Irani et al. (8) have reported that ras expression causes extracellular superoxide anion generation by a membrane-associated NADPH oxidase. This finding was confirmed for the ras-expressing cells used in our study, as the NO donor SNP mediated strong apoptosis induction by a reaction that was blocked by extracellular SOD. This is indicative of the generation of superoxide anions that have reacted with NO and have formed apoptosis-inducing peroxynitrite (17; Heigold...
et al., submitted for publication). The work of many groups shows that NO has no direct apoptosis potential and rather inhibits apoptosis through termination of lipid peroxidation (41–48). In contrast, peroxynitrite represents an efficient apoptosis inducer (17,31,49; Heigold et al., submitted for publication; for reviews see refs 18,50). As peroxynitrite formation is very fast and as peroxynitrite represents an efficient apoptosis inducer, this assay is rather sensitive. The specific involvement of superoxide anions is thereby proved by the complete inhibition of NO-mediated apoptosis induction in the presence of SOD. The minor, yet distinct, degree of leakiness of our induction system which was seen during clonal analysis of morphological transformation features was also confirmed for superoxide anion production. IR-1 cells without IPTG treatment showed weak apoptosis induction in the presence of NO, which was inhibited by SOD. In contrast, parental cells without the ras-containing expression plasmid showed no apoptotic reaction.

As ras-expressing IR cells, but not the uninduced control population of cells, were sensitive for intercellular induction of apoptosis, a causal relationship between expression of the transformed state and sensitivity for elimination by non-transformed neighbouring cells is proved. This selectivity seems to be the basis for the specific anti-oncogenic action of this novel potential control system of oncogenesis. Selectivity for apoptosis induction in transformed cells is based on superoxide anion production by transformed target cells. This conclusion is based on the following arguments. (i) ras-expressing cells generate extracellular superoxide anions, as demonstrated by sensitivity to NO-mediated apoptosis induction, which requires superoxide anions for peroxynitrite formation. (ii) Intercellular induction of apoptosis in ras-expressing cells by non-transformed fibroblasts is dependent on superoxide anions, as it is blocked by SOD. In line with the delineation of intercellular signalling by src-transformed cells, superoxide anions seem to be required for hydrogen peroxide generation through dismutation (therefore the reaction is blocked by catalase) and for interaction with HOCl generated by a novel peroxidase. This interaction causes the generation of apoptosis-inducing hydroxyl radicals. Therefore, this signalling pathway is blocked by the HOCl scavenger taurine and by the hydroxyl radical scavenger terephthalate. (iii) Recent reconstitution studies using IR cells with induced ras expression and purified myeloperoxidase demonstrated that transformed cells (in contrast to non-transformed cells) generate sufficient superoxide anions to drive myeloperoxidase-mediated apoptosis induction through HOCl formation (based on the hydrogen peroxide derived from superoxide anion dismutation) and hydroxyl radical formation (after interaction of HOCl with superoxide anions) (51). Therefore, superoxide anions selectively produced by ras-transformed cells determine efficiency and selectivity of intercellular induction of apoptosis.

The conclusions derived from the ras expression system link oncogene expression, subsequent establishment of the transformed phenotype and specific generation of superoxide anions by transformed cells directly to their sensitivity for intercellular induction of apoptosis. This sequence of events seems to be the crucial step that determines selectivity of intercellular induction of apoptosis. This important and central feature of intercellular induction of apoptosis has therefore been verified by several other parallel approaches which are in line with the results and interpretations presented here. (i) Revertants from src-transformed cells lost sensitivity together with the transformed phenotype (52). In contrast to src-transformed cells, the revertants did not generate extracellular superoxide anions, as they were no longer sensitive to NO-mediated apoptosis induction (Heigold et al., submitted for publication). (ii) NRK cells which exhibited reversible expression of the transformed phenotype after administration of EGF and TGF-β exhibited sensitivity to intercellular induction of apoptosis in parallel. This selective sensitivity depended on superoxide anions, as it was blocked by SOD (53). (iii) Fusion products between transformed and non-transformed cells lost sensitivity for intercellular induction of apoptosis together with the transformed phenotype. In parallel they lost extracellular superoxide anion production (Wilmsmeyer and Bauer, in preparation). Taken together, these data demonstrate the crucial role of transformed target cell-derived superoxide anions as determinants for selective apoptosis induction by non-transformed effector cells.

Besides establishing sensitivity for intercellular induction of apoptosis (a process that efficiently discriminates between transformed and non-transformed cells), ras expression also increased the sensitivity for apoptosis inducers like mitomycin C, ceramide and CHX, which are not selective for the transformed phenotype. Inhibition of protein synthesis by CHX has recently been shown to cause depletion of endogenous apoptosis inhibitory proteins (endogenous survival factors),
operationally defined inhibitors of a constitutively expressed apoptosis machinery (38). The time between addition of CHX can be taken as a correlate of the initial concentration of endogenous apoptosis inhibitory proteins. The faster apoptosis induction in ras-expressing cells compared to control cells can therefore be taken as an indication of a lower concentration of endogenous apoptosis inhibitory proteins. It should render Ras-expressing cells more sensitive to various apoptotic stimuli. It seems very likely that the increased sensitivity of ras-expressing cells to mitomycin C and ceramide is based on their lower content of endogenous inhibitors of apoptosis.

The biochemical nature of the endogenous inhibitors of apoptosis (endogenous survival factors) as defined in functional assays in this paper remains enigmatic. Based on the work of many other groups, two families of known apoptosis inhibitors might be considered as likely candidates: the bcl-2 family and the iap family (for reviews see refs 50,54). Whereas bcl-2 seems to act directly at the mitochondrial permeability transition pore, iap proteins inhibit caspase 3, which is activated by caspase 9 downstream of mitochondrial depolarization. As bcl-2 overexpression did not influence the kinetics of apoptosis after addition of CHX (Bauer, unpublished observation), bcl-2 can be excluded as an endogenous inhibitor of apoptosis as operationally defined in this paper. Since it has recently been shown that the intracellular iap concentration is decreased after inhibition of protein synthesis and that iap proteins exhibit a short chemical half-life (55), these might be the endogenous survival factors described here. In line with this hypothesis, apoptosis induction by CHX did not cause mitochondrial depolarization (Bauer, unpublished observation). This indicates that depletion of endogenous survival factors (possibly iap proteins) through inhibition of their de novo synthesis might allow direct activation of caspase 3 without prior mitochondrial depolarization. More experimental efforts are required to clarify this point.

However, the regulatory principle of endogenous apoptosis inhibitory proteins does not determine sensitivity of ras-expressing transformed cells for intercellular induction of apoptosis per se, as TGF-β treatment and ras expression had the same effect on endogenous apoptosis inhibitory proteins, but only ras expression caused sensitivity to intercellular induction of apoptosis. However, if ras-expressing, superoxide anion-generating cells are pretreated with TGF-β (a regimen that further decreases their concentration of endogenous apoptosis inhibitory proteins), the efficiency of intercellular induction of apoptosis was enhanced. Therefore, though not principally determining sensitivity for intercellular induction of apoptosis, endogenous apoptosis inhibitory proteins seem to have an additional modulatory effect on intercellular apoptosis induction. By analogy, a drastic increase in endogenous apoptosis inhibitory proteins has recently been shown to prevent intercellular induction of apoptosis and thus may be involved in the establishment of resistance during tumour formation (56,57).

It has to be pointed out that this study was performed with cells transformed in vitro which had not been challenged by natural anti-tumour systems. This strategy allows a direct study of ras-related regulation of apoptosis, without possible selection for resistant cells that is likely to occur in vivo. Therefore, the increased sensitivity for apoptosis induction of ras-transformed fibroblasts may not necessarily be found in ex vivo tumour cells expressing active Ras. Rather, it can be speculated that ras-expressing cells might acquire additional mechanisms that compensate for their increased sensitivity in order to become tumour-forming, thereby masking the primary effect of ras expression and allowing their survival despite the attack of natural anti-tumour systems.

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