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Bcl-2-Mediated Inhibition of Apoptosis Prevents Immunogenicity and Restores Tumorigenicity of Spontaneously Regressive Tumors[1]

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Tumor cell clones from a rat colon carcinoma differ in their tumorigenicity and immunogenicity. The PRO clones give rise to progressive tumors, whereas the REG clones yield tumors that regress in a few weeks through a specific immune response. REG cells were more sensitive than PRO cells to apoptosis triggered by serum withdrawal in vitro. Furthermore, a fraction of REG cells, but no PRO cells, underwent apoptosis in the hours following injection into syngeneic rats. To further analyze the role of apoptosis, we overexpressed the antiapoptotic protein Bcl-2 in REG cells. Unlike parental or fake-transfected REG cells, Bcl-2-overexpressing REG cells resisted serum withdrawal-induced apoptosis, did not undergo apoptosis at 48 h postinjection into naive syngeneic rats, and gave rise to progressive, metastatic, and lethal tumors. Interestingly, REG-bcl2 cells were rejected by syngeneic hosts that had been preimmunized by an injection of parental REG cells, indicating that Bcl-2 overexpression did not alter tumor cell sensitivity to the effector cells of the immune response. Taken together, these observations indicate that tumor cell apoptosis may contribute to immunogenicity. ***The Journal of Immunology,*** **1998, 161: 1433–1438.**

Most clinical and experimental cancers contain mutated or abnormally expressed proteins that could be recognized by the immune system and lead to tumor rejection. However, tumor cells are usually well-tolerated by their host, which leads to tumor progression, invasion, metastasis, and, ultimately, the death of the host. The few examples of spontaneously regressive tumors are interesting, as they point out mechanisms that could lead to a reversion of the customary progressive phenotype.

We previously found that tumor cell clones, which we had established from a chemically induced colon carcinoma growing in an inbred strain rat, constitutively differed in their tumorigenicity and immunogenicity in syngeneic hosts (1, 2). Several clones gave rise to tumors that grew continuously, produced distant metastases, and finally killed their host. These clones were named PRO, as they yielded progressive tumors. Other clones gave rise to tumors that progressed for 2 to 3 weeks, regressed, and completely disappeared. We showed that these clones, named REG for regressive, induced a strong tumor-specific immune response that fully and durably protected animals against a subsequent injection of PRO cells (3). The spontaneous regression of the REG tumors was related to this T cell-dependent immune response, since REG cells gave rise to progressive tumors in nude mice, cyclosporin-treated syngeneic rats (4), or rats in which a growing PRO tumor had induced a tumor-specific immune tolerance (3). These properties made the PRO/REG cell model well-suited for the study of tumor immunity.

In the present study, we show that the tumorigenicity and immunogenicity of the PRO and REG cell lines is related to their ability to undergo apoptosis when cultured in vitro and injected in vivo. Overexpression of the protooncogene *bcl-2* was shown to enhance cell survival following growth factor withdrawal (5) and to inhibit the apoptosis induced by a variety of stimuli (6-8). We also show that *bcl-2* overexpression decreases REG cell sensitivity to apoptosis and induces REG tumor progression in a syngeneic host. Bcl-2-transfected REG cells remain sensitive to a previously established antitumor immune response. These results suggest that tumor cell apoptosis contributes to the induction of a specific immune response in vivo.

Materials and Methods

Animals, cell lines, and tumorigenicity assays

The animals used in these experiments were syngeneic BD-IX strain rats that had been bred in our laboratory by brother-sister mating. The regressive variant DHD-K12/TSb (REGb) and the progressive variant DHD-K12/TRb (PROb), as well as other PRO and REG clones used in some experiments, were all established from the DHD tumor, which is a colon adenocarcinoma induced by 1.2-dimethylhydrazine in a BD-IX rat (1). These tumor cells were cultured in a mixture of Ham's F-10 medium, DMEM, and FBS (10:10:1 v/v, complete culture medium) as described previously (2). For the tumorigenicity assays, 1×10^6 tumor cells in 100 μl of serum-free Ham's F-10 medium were injected s.c. into the thoracic wall of syngeneic BD-IX rats. Tumor volume was evaluated weekly, using a caliper to measure two perpendicular diameters. Rats bearing tumors that were >6 ml were killed and examined for metastases.

Histologic study of the tumor cell injection site

Animals were killed at 6, 24, or 48 h after PRO or REG cell injection. The site of tumor cell injection was resected and either fixed in formaldehyde (4% in PBS) and embedded in paraffin or embedded in Tissue-Tek (Miles, Elkhart, IN) and snap-frozen in methylbutane that had been cooled in liquid

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nitrogen. Apoptotic cells were labeled on 5-μM paraffin-embedded sections according to the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)[3] procedure (9) using terminal transferase and biotin-16-2′-deoxyuridine-5′-triphosphate from Boehringer (Mannheim, Germany) and streptavidin-peroxidase (Amersham, Les Ullis, France), followed by hemalum staining. Tumor cells on slides stained with hemalum-eosin were distinguished from inflammatory cells according to their size, their large nuclei with oversized nucleoles, and their assembly in nodules. An immunohistochemical study of tumor-infiltrating inflammatory cells was performed in acetone-fixed 5-μM cryostat sections. Mouse mAbs to rat monocytes (ED1), class II MHC (OX-17), CD3 (R7/3), CD4 (W3/25), and CD8 (OX8), as well as an IgG isotype-matched control were obtained from Serotec (Oxford, U.K.). Mouse mAb against rat mature tissue macrophages (Ki-M2R) was a gift of Professor H. H. Wacker (Institute of Pathology; Kiel, Germany). Sections were incubated with mAb and biotinylated sheep Ab to mouse IgG (Amersham), subsequently incubated with streptavidin-peroxidase, and stained with aminoethylcarbazole. In other sections, tumor cells were stained after incubation with a rabbit polyclonal Ab to cytokeratin (Monosan, Uden, The Netherlands).

Cell sensitivity to apoptosis in vitro

The sensitivity of cultured cells to apoptosis was determined after serum withdrawal from the culture medium. Tumor cells (1×10^6 cells in 1 ml of complete medium) were seeded in the wells of 24-well flat-bottom microculture plates (Intermed, Nunc, Roskilde, Denmark) and cultured for 24 h. The wells were gently washed three times with serum-free Ham's F-10 medium and cultured in this medium for 72 h at 37°C in a 5% CO_2 atmosphere. At the end of this incubation period, floating cells were pooled with adherent cells that had been detached with EDTA-trypsin. The pooled cells were incubated for 18 h at 4°C in HBSS (Life Technologies, Paisley, U.K.) containing 2% formaldehyde and 10 μg/ml Hoechst 33258 bisbenzimide fluorochrome (Aldrich-Chemie, Steinheim, Germany). Next, the percentage of apoptotic cells was determined on an epifluorescence microscope (DMR, Leica, Wetzlar, Germany) at ×400 magnification. Apoptotic cells were identified by chromatin condensation or fragmentation that clearly distinguished them from mitotic cells. The percentage of apoptotic cells was determined in populations of ≥400 cells. To compare the cytotoxic effect of serum-free medium on the various PRO and REG clones, 2×10^4 tumor cells in 200 μl of complete medium were seeded in wells of 96-well flat-bottom microculture plates (Nunc) and cultured for 24 h. The medium was subsequently sucked up, and the wells were washed three times with serum-free medium and filled with either serum-free or 10% FBS-supplemented medium. Cells were cultured for 14 days at 37°C in a 5% CO_2 atmosphere. Serum-free and FBS-complemented medium was replaced every other day by homologous fresh medium. At the end of this incubation period, the culture medium was drained off, the wells were gently washed twice with PBS, and the cultures were fixed for 15 min with methanol. The plates were allowed to dry and then stained with 1% crystal violet per well for 30 min at room temperature. The plates were carefully washed under tap water and dried, and the residual stain was eluted in 100 μl of 33% acetic acid in water (v/v). The absorbance of each well was read at 570 nm on an Anthos scan (Labtec Instruments, Salzburg, Austria). Quadruplicate wells were used to determine the mean and SD.

Bcl-2 transfection of the REG cell line

REG cells were transfected with pEBS7 expression plasmid, which was either empty or contained cDNA-encoding human *bcl-2* (10). pEBS7 plasmid contains the hygromycin B phosphotransferase gene, which is a selectable marker for resistance to hygromycin. Transfections were performed using calcium phosphate precipitation procedures (11). The *bcl-2* or control transfectants were selected for 4 wk in complete medium supplemented with 1 mg/ml hygromycin (Sigma, St. Louis, MO). At such a concentration, hygromycin is lethal in 7 days for wild-type (wt) REG cells. Hygromycin-resistant cells were expanded and then cloned by limiting dilution. We selected REG clones with the highest Bcl-2 expression as determined by Western blot analysis. REG cells transfected with empty pEBS7 plasmid were used as a control.

Western blot analysis

After detachment with trypsin and EDTA, subconfluent cultured cells were lysed at 4°C for 30 min in lysis buffer (150 mM NaCl, 1 mM KH_2PO_4, 1 mM EGTA, 1 mM Na_3PO_4, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.15 U/ml aprotinin, 1 μg/ml pepstatin, and 10% glycerol) and centrifuged for 15 min at 15,000 × *g*. The protein concentration was measured in the supernatant using the micro bicinchoninic acid protein assay (Pierce, Asnieres, France). Equal amounts of proteins (50 μg) were separated by SDS-PAGE using a 12% polyacrylamide gel and were electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, Ivry-sur-Seine, France). Blots were blocked with 5% nonfat milk in PBS with 0.1% Tween 20. To control the effects of cell transfection, blots were probed with mouse mAb (1/1000) to human Bcl-2 (Dako, Glostrup, Denmark) or to rat Hsp90 (StressGen, Victoria, Canada). To determine the content of autochthonous proteins, blots were probed with rabbit polyclonal Ab (1/1000) reacting with rat rBcl-2 or Bax (Santa Cruz Biotechnology, Santa Cruz, CA). After three 10-min washes in PBS with 0.1% Tween 20, the blots were incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham). The Amersham enhanced chemiluminescence Western blotting analysis system was subsequently used for protein detection. The results are representative of three separate experiments.

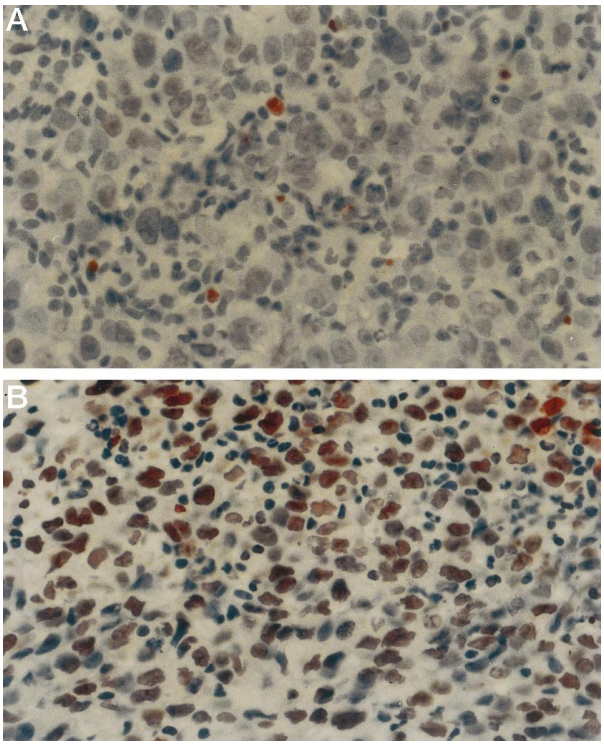

FIGURE 1. Comparison of apoptotic tumor cells at 48 h after a s.c. injection of 1×10^6 PRO (*A*) or REG (*B*) tumor cells. Apoptotic cells, which were identified as tumor cells according to their size and their intercellular attachment, were labeled in brown with the TUNEL assay. Both sections were taken at ×330 magnification.

Results

Tumor cell apoptosis after PRO and REG cell injection

As previously reported (12), PRO and REG cells give rise to measurable, histologically proven local tumors that progress until 14 to 18 days after s.c. injection. At this point, PRO tumors continue to progress, while REG tumors regress until their complete disappearance, which occurs at 4 to 6 wk postinjection. To analyze the reasons for this different evolution, we studied tumor foci during the 48 h after a s.c. injection of PRO or REG cells. When examined at 6 h postinjection, tumor cells from both lines were similarly preserved. PRO cells remained morphologically normal at 24 and 48 h postinjection, whereas numerous REG cells demonstrated condensed or fragmented chromatin and eosinophilic staining of the cytoplasm. These changes suggested cell apoptosis. Indeed, when the TUNEL assay was performed on sections of the tumor cell injection site, numerous apoptotic tumor cells were found in

[3] Abbreviations used in this paper: TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphospate nick-end labeling; wt, wild-type; c.i., confidence interval.

the REG cell foci, whereas apoptotic cells were absent or rare in the PRO cell foci (Fig. 1). We also examined inflammatory cells that infiltrate PRO and REG cell injection sites. These cells were rare in sections of sites that were resected 6 h after tumor cell injection but became abundant after 24 and 48 h. Polymorphonuclear cells were identified at 24 h after tumor cell injection but had almost entirely disappeared after 48 h. T cells remained rare in the tumor infiltrate up to 48 h after tumor cell injection. The most abundant infiltrating cells inside and around PRO and REG cell foci were round mononuclear cells that were labeled with ED1 mAb, a marker of rat monocytes and macrophages (13). These ED1-stained cells were also seen inside and around the blood vessels surrounding the tumor cell area, suggesting their blood origin. Inflammatory cells did not express class II MHC molecules at 24 h after tumor cell injection, whereas a fraction of these cells expressed this marker of APCs after 48 h. Unlike the mature resident macrophages found in the normal s.c. tissue, tumor-infiltrating inflammatory cells were not stained with Ki-M2R mAb. No significant difference was found between the PRO and REG cell injection sites with regard to the density and the type of infiltrating inflammatory cells at 24 and 48 h after tumor cell injection.

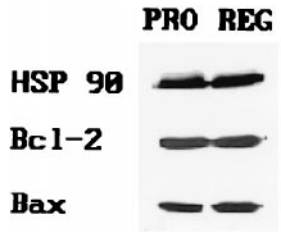

FIGURE 3. Western blot analysis of endogenous Bcl-2 and Bax proteins in REG and PRO cells. Cell extracts were analyzed using Abs against rat Bcl-2 and Bax; rat Hsp90 was used as a control of the amount of deposited extracts.

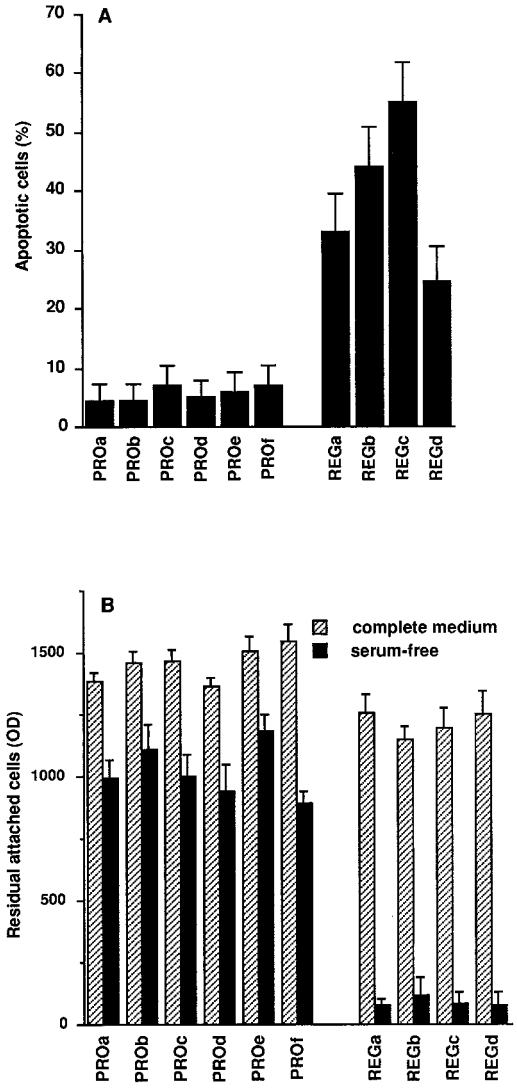

FIGURE 2. Comparison of six PRO clones and four REG clones with regard to their sensitivity to apoptosis when cultured in serum-free medium. Confluent monolayers were established in culture medium that had been supplemented with 5% FBS before serum withdrawal. *A*, Following a 3-day, serum-free culture, the percentage of apoptotic cells in pooled floating cells and adherent cells was determined after chromatin staining with Hoechst 33258. Each count was established on 200 or 400 cells. The 95% c.i. is indicated by a bar. *B*, After a 14-day culture in medium that had been supplemented with 10% FBS (hatched columns) or serum-free medium (solid columns), the residual attached cells were washed, fixed with methanol, and stained with crystal violet. Cell survival was estimated by measuring the OD of eluted crystal violet in arbitrary units. SD is indicated by a bar.

REG cells are more sensitive than PRO cells to apoptosis when cultured in serum-free medium

To determine whether the high rate of apoptosis observed in REG cells relative to PRO cells after s.c. injection was due to intrinsic properties of the cells or to an early reaction of the host, PRO and REG cells were compared with regard to their sensitivity to apoptosis when cultured in vitro as two-dimensional monolayers, with or without FBS supplementation. The deprivation of growth factors in serum-free medium is known to induce the apoptosis of sensitive cells. We compared six PRO clones and four REG clones that were independently obtained from the same colon cancer cell line and yielded progressive or regressive tumors, respectively, when injected into syngeneic hosts (2). The percentage of apoptotic cells was low and nearly the same in PRO and REG clones when cultured for 3 days in FBS-supplemented medium. REG clones were more sensitive than PRO clones to apoptosis induced through a 3-day culture in serum-free medium. The rate of apoptotic cells, as determined after Hoechst 33258 staining, was higher in the REG clones than in the PRO clones (Fig. 2*A*). Numerous apoptotic cells and cellular debris were observed floating in the medium above the serum-deprived REG clones. When apoptotic cells and cellular debris were collected daily for 4 days by centrifugation of the serum-free culture medium and measured as proteins, they were found to be seven times more abundant in REG than in PRO cell supernatant. We also observed that the density of residual adherent cells was strongly reduced when the four REG clones had been cultured for 14 days in serum-free medium, whereas this treatment had only a limited effect on the six PRO clones (Fig. 2*B*).

REG cells overexpressing human bcl-2 *gave rise to progressive and metastatic tumors*

Western blot analysis was performed on REG and PRO cell extracts using Abs against rat antiapoptotic Bcl-2 and proapoptotic Bax proteins. REG and PRO cells expressed these endogenous proteins in comparable amounts (Fig. 3). To induce Bcl-2 overexpression, REG cells were transfected with either empty or human *bcl-2* cDNA-containing pEBS7 plasmid. Several clones of hygromycin-resistant transfected cells were obtained. Two clones of

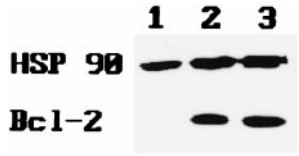

FIGURE 4. Western blot analysis of human Bcl-2 protein in transfected cells. REG cells were transfected with an empty pEBS7 vector (REG-hygro) or with cDNA-encoding human Bcl-2 using the calcium phosphate method. Cell lysates from empty plasmid- or Bcl-2-transfected REG cells (two clones: Bcl-2-D7 and Bcl-2-E8) were studied by Western blot analysis using a specific mAb against human Bcl-2. Lanes represent the following cells: 1, REG-hygro; 2, REG-bcl2-D7; 3, REG-bcl2-E8.

REG cells that had been transfected with *bcl-2* cDNA were selected for their high expression of the human Bcl-2 protein, as detected by Western blot analysis using a human-specific anti-Bcl-2 Ab (Fig. 4). This expression was checked to ensure that it remained stable after multiple passages in vitro. These clones were called REG-bcl2 cells. One of the REG clones that had been transfected with the empty vector (REG-hygro) was used as control. REG-bcl2 cells were resistant to the apoptosis induced by serum-withdrawal (13% apoptotic cells after a 3-day depletion, 95% confidence interval (c.i.): 4.3%) when compared with REG-hygro cells (35% apoptotic cells, 95% c.i.: 4.7%) (Fig. 5). We used the TUNEL assay to identify apoptotic cells on sections of the tumors yielded by transfected REG cells into syngeneic rats. No apoptotic tumor cells were observed at the injection site where REG-bcl2 cells had been administered 48 h before, whereas REG-hygro nodules contained a mixture of apoptotic and nonapoptotic tumor cells (Fig. 6). wt REG and REG-hygro cells gave rise to tumors that completely regressed in 3 to 4 wk. In contrast, continuously progressive tumors were observed in 14 of 18 injected rats in three separate experiments with two different REG-bcl2 clones (Fig. 7). Although REG-bcl2 tumors grew about twice as slowly as PRO tumors, they progressed to large volume tumors. Histologically proved metastases to axillary and mediastinal lymph nodes and

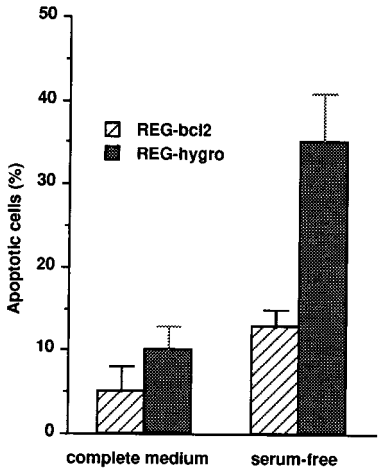

FIGURE 5. Comparison of REG-hygro and REG-bcl2 with regard to their sensitivity to apoptosis when cultured in serum-free medium. Confluent monolayers were established in culture medium that had been supplemented with 5% FBS before serum withdrawal. Following a 3-day, serum-free culture, the percentage of apoptotic cells in pooled adherent and floating cells was determined after chromatin staining with Hoechst 33258. REG-bcl2 cells were resistant to the apoptosis induced by serum withdrawal compared with REG-hygro cells. Each count was established on 200 or 400 cells. The 95% c.i. is indicated by a bar.

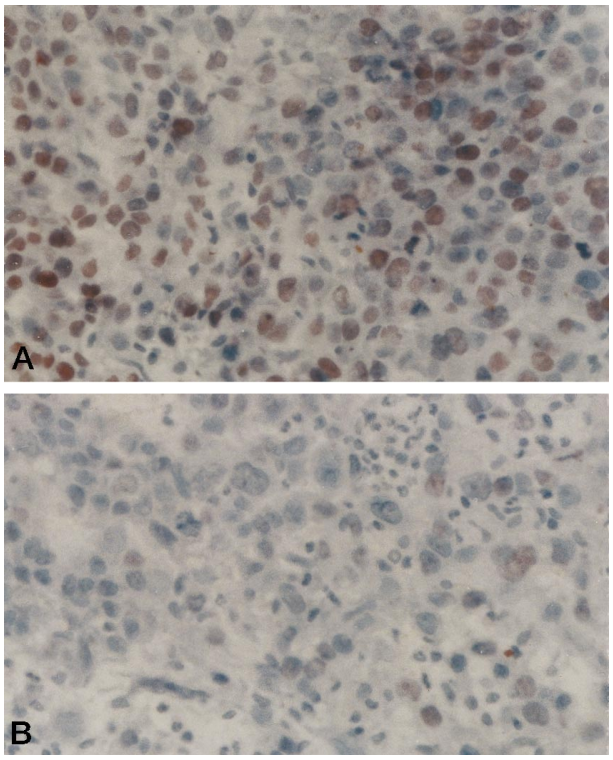

FIGURE 6. Comparison of apoptotic tumor cells at 48 h after a s.c. injection of 1×10^6 REG-hygro (*A*) or REG-bcl2 (*B*) tumor cells. Apoptotic cells, which were identified as tumor cells according to their size and their intercellular attachment, were labeled in brown with the TUNEL assay. Both sections were taken at ×330 magnification.

several micrometastases to the lung were found in two rats bearing a voluminous s.c. tumor that were killed at 126 days after REG-bcl2 cell injection. Furthermore, fragments from REG-bcl2 resected tumors grew as a progressive tumor when transplanted into naive syngeneic recipients. Most of the cancer cells in these REG-bcl2 tumors still expressed human Bcl-2 on immunohistologically

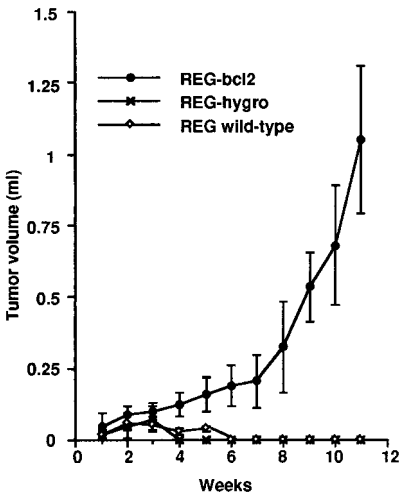

FIGURE 7. Growth curves of the tumors after a s.c. injection of 1×10^6 REG, REG-hygro, and REG-bcl2 cells into naive syngeneic rats. The curves represent the mean of three independent experiments. SD is indicated by a bar. All of the injections with wt REG and REG-hygro cells gave rise to tumors that completely regressed in 3 to 4 wk. A total of 14 of 18 injected rats with REG-bcl2 cells gave rise to progressive tumors. The line REG-bcl2 (●) represents the mean of the growth of these 14 tumors.

stained sections, demonstrating the in vivo stability of Bcl-2 overexpression (data not shown).

REG-bcl2 cells were rejected in rats immunized against wt REG cells

To determine whether the progression of REG-bcl2 tumors was due to their resistance to the effector mechanisms of antitumor immunity, REG-bcl2 cells were injected into naive rats and into rats that had been immunized with three monthly injections of 1×10^6 wt REG cells. REG-bcl2 cells gave rise to continuously progressive tumors in five of six naive animals, while no tumors appeared in the six preimmunized rats. These observations indicate that REG-bcl2 cells are rejected by the immune system despite Bcl-2 overexpression, suggesting that the progression of REG-bcl2 tumors is not the consequence of tumor cell resistance to immune rejection.

Discussion

In the present study, we found that a portion of the REG cells, but very few PRO cells, underwent apoptosis in the 2 days following their s.c. injection into syngeneic rats. REG tumor regression is not a direct consequence of this apoptosis, which is restricted to a limited fraction of REG cells. Apoptosis does not prevent the provisional growth of REG tumors that are palpable, measurable, and histologically documented between 6 and 18 days after tumor cell injection (12). REG tumor regression occurs later on, as a consequence of an established immune response. We also found that different REG clones were more sensitive to apoptosis than different PRO clones when these clones were cultured in serum-free medium, a condition that is known to enhance apoptosis through the depletion of exogenous growth factors (14, 15).

To investigate a possible relationship between apoptosis and the terminal regression and healing of REG tumors, we enhanced REG cell resistance to apoptosis by overexpressing Bcl-2, an apoptosis-suppressing protein. The clones that overexpressed Bcl-2 were more resistant to apoptosis in vivo and in vitro and gave rise to progressive, metastatic, and lethal tumors. Bcl-2 overexpression might either enhance the resistance of tumor cells to an established immune response or decrease the capability of tumor cells to induce this response. Our observation that REG-bcl2 cells were rejected in rats that had been preimmunized against wt REG cells is more in keeping with the second possibility. Bcl-2 transfection reportedly does not protect cells from lysis mediated by CTLs or activated NK cells (16). We have previously shown that PRO cells were also rejected in rats that had been previously immunized against REG cells (3). This finding suggests that the resistance of REG-bcl2 and PRO cells to apoptosis decreases their capability to induce an immune response rather than their sensitivity to the effectors generated by this response.

The induction of a relative resistance to apoptosis after *bcl-2* transfection was sufficient to prevent a regression of the REG tumors. Thus, REG-bcl2 cells are fairly similar to PRO cells, which are relatively resistant to apoptosis, are poorly or nonimmunogenic when injected into syngeneic hosts, and are able to give rise to progressive and lethal tumors. PRO and REG cells express similar levels of endogenous Bcl-2 and Bax. Bax is a Bcl-2 family protein that heterodimerizes with Bcl-2, neutralizes its apoptosis-protecting property (17), and can suppress tumorigenesis in other tumor models (18). Thus, wt PRO and REG cells differ in their sensibility to apoptosis through other still undetermined mechanisms.

PRO and REG cells express comparable levels of class I MHC on their plasma membrane but do not express class II MHC nor B7 costimulatory molecules and are not capable of directly presenting their Ags to T cells (data not shown). To induce an immune response, tumor Ags have to be presented to T cells by professional APCs (19), preferentially inside of the lymph nodes or the spleen (20). Unlike PRO cells, apoptosing REG cells release a great amount of cellular debris in the culture medium, and the same release is likely to occur in vivo. This cellular debris is an efficient source of Ag for T cell activation (21-23). The cellular debris of apoptotic cells, called apoptotic bodies, is still enclosed by a cell membrane. However, this membrane differs from that of healthy cells, since phosphatidylserine is aberrantly exposed on its outer leaflet and enhances recognition and internalization by phagocytic cells (24). We found that apoptotic cells and the debris released by serum-deprived REG cells were labeled with FITC-conjugated annexin V, a ligand for surface-exposed phosphatidylserine (data not shown). The phosphatidylserine receptor is more strongly expressed on monocytes than on mature macrophages (24). Thus, apoptotic bodies are likely to be recognized and ingested by the abundant monocytes that infiltrate the tumor cell foci early after cell injection. These monocytes strongly express ED1, a marker of phagolysosomes that is correlated with the phagocytic activity of these cells (25). Inflammatory monocytes and macrophages have the capacity to migrate into the secondary lymphoid tissues (26), in which they can deliver antigenic material from ingested particles to dendritic cells for presentation to T cells (27).

The PRO and REG cell variants have been isolated from the same tumor cell culture. Unlike other nontumorigenic or regressive cell variants (28), REG cells did not result from a mutagenic treatment inducing highly immunogenic protein mutations. Despite their dramatic difference in immunogenicity and tumorigenicity, PRO and REG cells share a similar antigenic profile, since PRO cells are regularly rejected in hosts preimmunized with REG cells (3). The most striking finding of the present study was that resistance to apoptosis through Bcl-2 overexpression was sufficient to restore a progressive and lethal phenotype to tumor cell variants that constitutively yielded immunogenic and regressive tumors in all cases. This finding indicates that tumor cell apoptosis may be an important factor for the triggering of an effective immune response against cancer.

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