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Bcl-2 Protooncogene Expression in Cervical Carcinoma Cell Lines Containing Inactive p53

Xiao Huan Liang, Salvatore Mungal, Andrea Ayscue, John D. Meissner, Pawel Wodnicki, David Hockenbery, Stephen Lockett, and Brian Herman

Laboratories for Cell Biology, and Department of Cell Biology and Anatomy (X.H.L., S.M., A.A., J.D.M., P.W., B.H.), Lineberger Comprehensive Cancer Center (B.H.), School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599; Fred Hutchinson Cancer Research Center, Seattle, Washington (D.H.); Life Sciences Division, Lawrence Berkeley Laboratories, Berkeley, California 94720 (S.L.)

Abstract Bcl-2 protein expression has been found to block apoptosis and its overexpression has been implicated in lymphoid malignancies where the chromosomal translocation t(14;18) is present. In this study we investigated bcl-2 transcription and protein expression in cultured cervical carcinoma cell lines and keratinocytes. Western blotting and immunofluorescence microscopy demonstrated bcl-2 expression in the cytoplasm of 4 out of 5 cervical carcinoma cell lines examined (HeLa, CaSki, C-33A, and HT-3, but not SiHa). Bcl-2 protein expression was undetectable in normal keratinocytes. None of the cell lines examined demonstrated chromosomal translocation or rearrangement at the major breakpoint-cluster region (MBR) of the bcl-2 gene using either Southern blot or polymerase chain reaction (PCR) analyses. Northern blot analysis demonstrated low levels of bcl-2 transcription in HeLa, CaSki, and C-33A cell lines while reverse transcriptase (RT)-PCR demonstrated bcl-2 transcription in all cervical carcinoma cell lines which had bcl-2 protein expression. Thus, these data suggest that bcl-2 expression occurs in cervical carcinoma cell lines in the absence of chromosomal translocation or rearrangement of the bcl-2 gene. However, each of these cervical carcinoma cell lines contains inactive p53, either due to mutation (C-33A and HT-3) or via complexation and degradation with human papillomavirus (HPV) 16/18 E6 protein (HeLa and CaSki). Thus, functional p53, which can induce apoptosis in certain cells, is not present in these cervical cells which have increased bcl-2 expression. Increased bcl-2 expression under conditions of p53 inactivation may provide cells with a selective advantage for survival and consequently play a role in the development of cervical carcinogenesis.

Key words: bcl-2, p53, HPV, cervical carcinoma, apoptosis

In recent years, the cause of cancer has come to be viewed as not only a consequence of uncontrolled cell growth, but may also be due to loss of growth suppressing activities. An example of the latter is cervical cancer, one of the most common and fatal malignancies in women worldwide. Substantial evidence has been gathered linking cervical cancer to infection with certain genotypes of the human papillomaviruses (HPVs). Proteins encoded by early genes E6 and E7 of high risk genotypes HPV (e.g., 16/18) have been shown to possess transformation properties, and have been designated as oncoproteins [Zur Hausen, 1991; Howley, 1991]. Although the contribution of E6 and E7 to development of cervical neoplasia remains largely unknown, it has become evident that p53 and RB gene products complex with the E6 and E7 proteins, respectively, resulting in loss of their tumor suppressor functions [Werness et al., 1990; Dyson et al., 1989]. Recently, we have observed a physical complex between HPV E6 and p53 proteins in the cytoplasm of cervical cancer cell lines containing integrated HPV 16/18 [Liang et al., 1993]. This observation supports the hypotheses that complex formation prevents p53 from entering the nucleus of cells and consequent loss of p53 tumor suppressor activity.

The p53 gene product appears to have multiple functions. p53 can cause cell cycle arrest in response to DNA damage, allowing DNA repair

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Address reprint requests to Dr. Brian Herman, Department of Cell Biology and Anatomy, CB #7090, 232 Taylor Hall, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

before further progression through the cell cycle [Zambetti and Levine, 1993]. p53 has recently been found to induce the synthesis of waf-1/cip-1, a 21 kDa protein that binds to cell cycle dependent kinases (CDKs), leading to inactivation of the kinases and suppression of cell growth [El-Deiry et al., 1993; Harper et al., 1993]. In certain cells, p53 can trigger apoptosis [Yonish et al., 1991; Lowe et al., 1993; Ramqvist et al., 1993]. Recent evidence has been presented which suggests that certain viral oncoproteins such as adenovirus E1B 19 Kd protein can inhibit apoptosis, by affecting p53 activity [Debbas and White, 1993]. Thus, in cells lacking functional p53, apoptosis may be inhibited, but the mechanisms which regulate this antiapoptotic activity are not understood.

Bcl-2 (B-cell lymphoma/leukemia-2) is a new type of oncogene which has been found to contribute to transformation by blocking apoptosis, rather than by accelerating cell proliferation [Hockenbery et al., 1990]. The bcl-2 protooncogene was initially discovered due to a translocation between chromosome 14 and 18, and has been found in more than 80% of follicular B-cell lymphomas [Yunis et al., 1982]. The t(14;18) translocation juxtaposes the bcl-2 gene from chromosome 18q21 to the immunoglobulin heavy chain gene (IgH) locus on 14q32, leading to aberrant levels of bcl-2 expression in B cells [Tsujimoto et al., 1985a,b; Tsujimoto and Croce, 1986]. Increased bcl-2 expression has also been reported in a limited number of nonlymphoid cancers, e.g., neuroblastoma [Reed et al., 1991; Castle et al., 1993], prostate carcinoma [McDonnell et al., 1992], nasopharyngeal carcinoma [Lu et al., 1993], lung carcinoma [Ikegaki et al., 1994], and breast cancer [Haldar et al., 1994]. Epstein-Barr virus (EBV) LMP1 protein also inhibits apoptosis under conditions where increased bcl-2 expression has been found [Henderson et al., 1991]. Thus, viral infection may lead to cellular transformation through inactivation of p53 activity and/or up-regulation of bcl-2 expression. As cervical cancer is thought to be due to viral infection resulting in p53 inactivation, we examined bcl-2 expression in cervical carcinoma cell lines containing inactive p53, either due to mutation or complexation with HPV E6 protein. Our data indicate that p53 inactivation is associated with increased bcl-2 expression, which may contribute to transformation of cervical epithelial cells.

MATERIALS AND METHODS

Cell Culture

Normal human epidermal keratinocytes were obtained from Clonetics Corporation (San Diego, CA). The Raji Burkitt lymphoma cell line, as well as the cervical carcinoma cell lines HeLa, CaSki, SiHa, HT-3, and C-33A were obtained from the American Type Culture Collection (Rockville, MD). Keratinocytes were maintained in KGM medium (Clonetics Corporation), Raji and CaSki cells in RPMI/10% fetal bovine serum (FBS), HeLa, SiHa, and C-33A in MEM/10% FBS, and HT-3 in McCoy 5A/10% FBS.

Antibodies

The hamster 6c8 anti-human bcl-2 monoclonal antibody which was used in immunofluorescent and immunoblot analysis has been previously characterized and shown to be specific for the detection of bcl-2 protein [Hockenbery et al., 1990]. Rabbit anti-human bcl-2 polyclonal antibody (PharMingen, San Diego, CA) produced against a synthetic cysteine containing peptide (aa 41-54) of the human 239 amino acid bcl-2 protein [Tsujimoto and Croce, 1986] was also used for immunofluorescence and immunocompetition experiments.

Immunoblot Analysis

Approximately 2×10^7 cultured cells were washed twice in phosphate-buffered saline (PBS) and suspended in 0.5 ml lysis buffer (1% Triton X-100, 0.15 M NaCl, 10 μM Tris [pH 7.5]; phenylmethylsulfonyl fluoride [PMSF], 50 μg/ml; aprotinin, 50 μg/ml; and 50 μM leupeptin). The lysate was boiled in ¼ volume of 4 × protein loading buffer (60 μM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.002% bromphenol blue) for 10 min, centrifuged for 2 min, and 40 μl of each protein preparation was electrophoretically separated on 12% SDS-PAGE gel and subsequently transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). Membranes were blocked in Tris-buffered saline (TBS; 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl) containing 0.5% bovine serum albumin (BSA) and 1 μM PMSF for 2 h. Membranes were then incubated at room temperature with 6c8 hamster bcl-2 monoclonal antibody (1:100) for 1 h, washed 3 times with TBS, and incubated with alkaline phosphatase-conjugated rabbit anti-hamster IgG (1:1,500). The positive reaction was visualized with NBT and BCIP (Bio-Rad). Bcl-2 protein

expressed in baculovirus was used as a standard (PharMingen).

Immunofluorescence Analysis

Cells grown on coverslips were washed with PBS and fixed with cold acetone for 5 min. For indirect immunofluorescence, 6c8 hamster monoclonal (1:20) or rabbit polyclonal (1:200) bcl-2 antibodies were diluted with 3% BSA and cells were incubated with the antibodies for 30 min at room temperature. Controls were incubated with 10% goat serum (diluted in 3% BSA). Following a PBS rinse, staining with FITC goat anti-hamster IgG (1:100) or rhodamine goat anti-rabbit IgG (1:160) was performed for 30 min at room temperature. The coverslips were rinsed and then incubated with 0.2 μg/ml 4′, 6-diamidino-2-phenylindole hydrochloride (DAPI) (Molecular Probes, Eugene, OR) for nuclear staining, and then mounted on glass slides with crystal/mount. Bcl-2 staining was observed with an epifluoresence microscope coupled to a color CCD (American Innovision, San Diego, CA) or a monochrome CCD Xillix camera (Xillix Technologies Corp., Vancouver, Canada) and a digital image analysis system [Lockett et al., 1991].

DNA Extraction and Southern Hybridization

Genomic DNA was isolated from 90% confluent cultured cells by first washing two times in PBS, followed by lysis in 25 μM Tris-HCl, pH 8, 8.3 μM EDTA, 1% Laurylsarcosine. Lysates were incubated overnight with 200 μg/ml proteinase K at 50°C. Following two rounds of extraction with phenol/chloroform/isoamylalcohol (25:24:1) and one round of chloroform/isoamylalcohol (49:1), DNA was precipitated with 1/20 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold ethanol (−70°C) for 30 min, washed with 75% ethanol, vacuum dried, and dissolved in TE buffer (10 μM Tris-HCl, pH 8, 1 μM EDTA).

For Southern blot analysis, 10 μg of DNA was digested with 50 U of the DNA restriction enzyme EcoR I or Hind III. DNA fragments were separated on 1.0% agarose gel and transferred to nitrocellulose membranes (Bio-Rad). Lambda DNA digested with Hind III was used as a molecular weight marker. The follicular lymphoma cell line SU-DHL-4 DNA (Oncogene Science, Manhasset, NY) was used as positive control for translocation/rearrangement at the major breakpoint-cluster region (mbr) of the bcl-2 gene. Normal placental tissue DNA (Oncogene Science) was used as a control for non-rearranged bcl-2 gene. A 2.7 kb EcoR I/Hind III sliced fragment of human germline bcl-2 DNA (which included the mbr region; Oncogene Science), was labeled with α ^{32}P-dCTP (Amersham Life Science, Arlington Heights, IL) using a random priming kit (Boehringer Mannheim, Germany) to a specific activity of 3–5 × 10^8 cpm/μg, and was used as probe for Southern hybridization. Prehybridization and hybridization were performed at 42°C for 3–5 h and 20 h, respectively, according to Wahl et al. [1979]. After hybridization, membranes were washed 3 times in 2 × SSC/0.1% SDS at room temperature followed by 1–2 washes in 0.2 × SSC/0.1% SDS at 52°C for 30 min. Southern blots were then exposed to X-Omat AR film (Kodak, Rochester, NY) at −70°C for 4–7 days.

Polymerase Chain Reaction (PCR)

DNA was extracted as described above. PCR TransPrimers include 2 sets of oligonucleotides for both normal bcl-2 (MBR-1 and MBR-3) and bcl-2/IgH-JH translocation (MBR-1 and JH-1) alleles (Oncogene Science; Fig. 1). DNA from the follicular lymphoma SU-DHL-6 cell line (which contains the bcl-2/JH fusion gene) and keratinocytes were used as controls to document rearrangement and normal bcl-2 gene organization, respectively. AmpliWax PCR Gem (Perkin-Elmer Corp., Norwalk, CT) was added to each PCR tube containing 25 pmol/μl of either MBR-1/MBR-3 or MBR-1/JH-1 primer sets and melted at 80°C for 5 min. After cooling, the wax formed a solid layer on the primers. Subsequently, 500 ng of each DNA sample was added in a 50 μl PCR reaction mixture which contained Taq buffer (including 1.5 μM $MgCl_2$), 250 μM of each dNTP, and 2.5 U of Taq DNA polymerase (Boehringer Mannheim). The following thermal profile was performed on a Programmable Thermal Controller (MJ Research, Inc., Watertown, MA): denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 1.5 min. After 35 cycles of amplification, the final elongation step was prolonged to 10 min. Ten microliters of each PCR product was electrophoresed on 2% agarose gel and stained with ethidium bromide. Detection of the amplified products was carried out using Southern blot and a MBR-2 oligonucleotide probe (Oncogene Science; Fig. 1). The MBR-2 oligonucleotide was labeled using α^{32}P-dCTP and terminal transfer-

ase (Boehringer Mannheim) at 37°C for 15 min and was separated from unincorporated nucleotides by size exclusion chromatography through a Chroma spin-30 column (Clontech, Palo Alto CA). ExpressHyb solution (Clontech) was used in prehybridization and hybridization at 68°C for 30 min and 1 h, respectively. Following a high stringency wash at 65°C, the blot was exposed to X-ray film (Fuji Photo Film, Japan) for 90 min.

RNA Extraction and Northern Blot

Total RNA was extracted from 90% confluent cultured cells by two washes with PBS followed by suspension in solution D (4 M guanidinium thiocyanate, 25 μM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 μM 2-mercaptoethanol, 0.2 M 'ium acetate [pH 4], phenol, and chloroform).1:1:0.2]). Cell extracts were centrifuged at 00 rpm for 20 min and RNA precipitated m aqueous supernatant by adding an equiva- t volume of isopropanol. The pellet was again racted with solution D, precipitated with iso- ıpanol, washed with 75% ethanol, and vacuum ..ıed. RNA was dissolved in H_2O and stored at −70°C. For mRNA extraction, cells were suspended in lysis buffer containing 10 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 5 mM KCl, and 1% Triton X-100. mRNA was purified using Dynabeads Oligo $(dT)_{25}$ (Dynal, Lake Success, NY) as recommended by the manufacturer. For Northern blot analysis, 20 μg of RNA or 1 μg of mRNA samples were brought up to 25 μl buffer containing 37% formaldehyde, formamide, and 10 × MOPS (1.75/5/1) and incubated 15 min at 55°C. RNA was electrophoresed on 1% agarose, transferred to nylon membranes (Schleicher & Schuell, Keene, NH) and hybridization was performed at 42°C with α^{32}P-dCTP labeled bcl-2 DNA probe as described above for Southern hybridization.

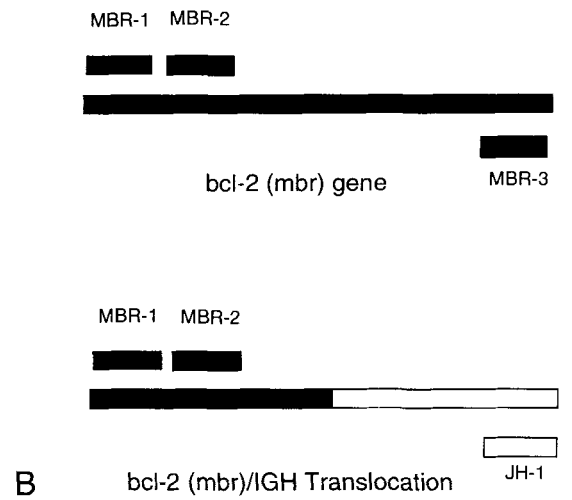

Fig. 1. Oligonucleotides used in PCR and Southern analyses. **A:** PCR amplification of the normal, non-rearranged bcl-2 (mbr) allele. MBR-1 and MBR-3: the sense and the anti-sense primers from chromosome 18 bcl-2 mbr region. MBR-2: the downstream oligonucleotide of MBR-1 used for Southern detection. **B:** PCR amplification of the bcl-2 (mbr)/IGH(JH) translocation allele. JH-1: anti-sense primer, from chromosome 14 immunoglobulin heavy chain HJ1 region.

RNA Reverse Transcription and Amplification (RT-PCR)

To eliminate residual genomic DNA from the RNA samples, 1 μg of RNA was treated with 1 U DNase (amplification grade; GibcoBRL, Gaithersburg, MD) for 15 min at 37°C, which was terminated by addition of 1 μl of 20 μM EDTA and incubation for 15 min at 65°C. For cDNA synthesis, we used a reverse transcription system (GibcoBRL). DNase treated RNA (1 μg) was incubated with 0.5 μg oligo(dT)12-18 at 70°C for 10 min, chilled on ice, and brought up to 20 μl of reaction volume in buffer containing 200 U RNase H^- MMLV reverse transcriptase (RT). The reaction was carried out at room temperature for 10 min, 42°C for 50 min, 70°C for 15 min, and then chilled on ice as recommended by the manufacturer. The mRNA template was degraded with 1 μl of RNase H at 37°C for 20 min.

First strand cDNA was amplified using PCR. Oligonucleotides were synthesized according to Finke et al. [1992] and from the published bcl-2 mRNA sequence: sense primer from nucleotides 333-357; anti-sense primer from nucleotides 652-628. Two microliters of cDNA product (derived from 100 ng RNA) was used as template. The reaction conditions and thermal profile were the same as described for DNA PCR. Ten microliters of each amplified product was subjected to electrophoresis on 2% agarose gels, transferred to BA-S nitrocellulose membrane (Schleicher & Schuell), and hybridized with a 3′ tail labeled bcl-2 detection antisense (DA) oligonucleotide (nucleotides 553-529). The methods of probe labeling, purification, and hybridization were the same as above for PCR. One microgram of DNase treated RNA sample was used as a control to document that all genomic DNA had been eliminated; the reaction was repeated but without reverse transcriptase.

RESULTS

Bcl-2 Protein Expression and Distribution in Human Cervical Carcinoma Cell Lines

The specificity of the 6c8 hamster bcl-2 monoclonal antibody has been previously described [Hockenbery et al., 1990]. With this antibody, we detected bcl-2 protein expression in protein lysates from each of the cervical carcinoma cell lines by Western blot analysis (Fig. 2). Baculovirus expressed bcl-2 protein was detected as a 26 kDa band; a band of the identical size was found in the Raji Burkitt lymphoma cell line, as well as in 4 of 5 cervical carcinoma cell lines HeLa, CaSki, HT-3, and C-33A. No bcl-2 protein was detected in SiHa or normal keratinocytes.

Bcl-2 protein has been reported predominantly in a membrane-associated cytoplasmic pattern including perinuclear, endoplasmic reticulum, and mitochondrial membranes [Hockenbery et al., 1990, 1991; Chen and Cleary, 1990; Monaghan et al., 1992], although a recent report has indicated chromosomal localization as well [Lu et al., 1994]. In this study, we examined the subcellular localization of bcl-2 in cervical carcinoma cells and keratinocytes. Consistent with immunoblotting, indirect immunofluorescent analysis using either monoclonal or polyclonal anti-human bcl-2 antibodies demonstrated bcl-2 protein expression in HeLa, CaSki, C-33A, and HT-3 cervical carcinoma cells (Fig. 3). Bcl-2 staining was distributed mainly in the cytoplasm of cells and, based on the intensity of staining, was greater in CaSki and HT-3 cells and lower in HeLa and C-33A cells. SiHa cells and normal keratinocytes had no detectable bcl-2 protein expression as judged by lack of detectable staining in the cytoplasm. Punctate nuclear staining (which may represent nucleoli) was also seen when the monoclonal bcl-2 antibody was employed (Fig. 3), but not with polyclonal bcl-2 antibody (Fig. 4). Whether this nucleolar staining is real or an artifact is difficult to determine; however, due to the fact that we did not observe nucleolar staining with the polyclonal anti-bcl-2 antibody and that a recent study demonstrates that bcl-2 can delay apoptosis in cytoplasts devoid of nuclei, if bcl-2 is localized to the nucleus, it may not be functionally important for bcl-2's anti-apoptotic activity [Jacobson et al., 1994].

To confirm the specificity of the polyclonal bcl-2 antibody, immunocompetition experiments were performed. Figure 4A demonstrates bcl-2

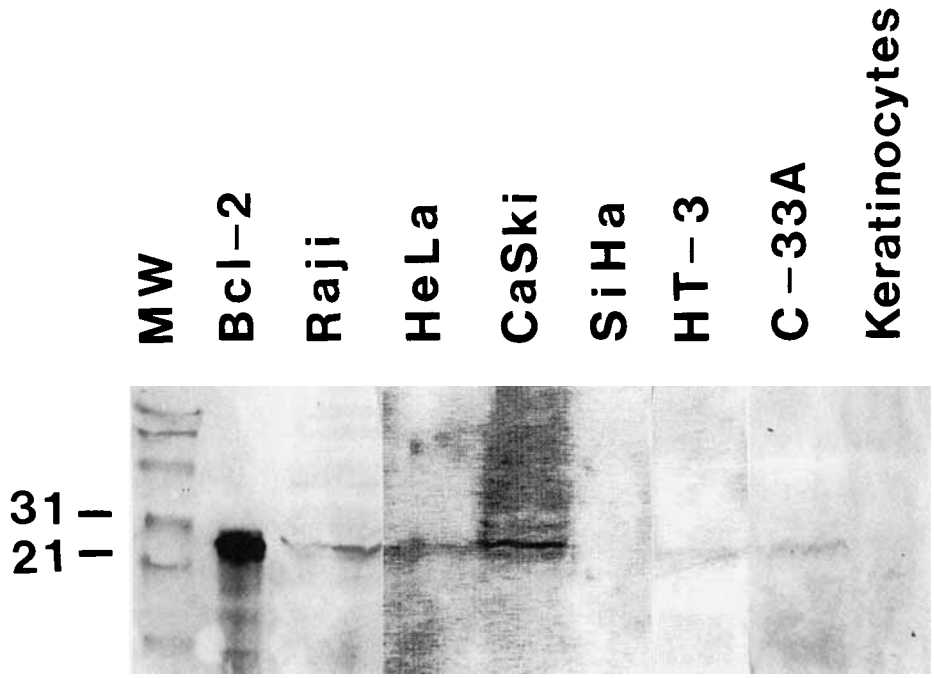

Fig. 2. Western blot analysis of bcl-2 protein expression in cervical carcinoma cells. Protein extracts from the different cells were separated by SDS-PAGE (12%) and transferred to nitrocellulose membrane. Bcl-2 protein was detected with 6c8 hamster bcl-2 monoclonal antibody (1:100 dilution), followed by alkaline phosphatase-conjugated rabbit anti-hamster IgG (1:1,500 dilution). A 26 kD protein expressed in baculovirus or Raji cells was used as bcl-2 protein standard. Molecular weight indicators (in kD) of protein marker are given at left.

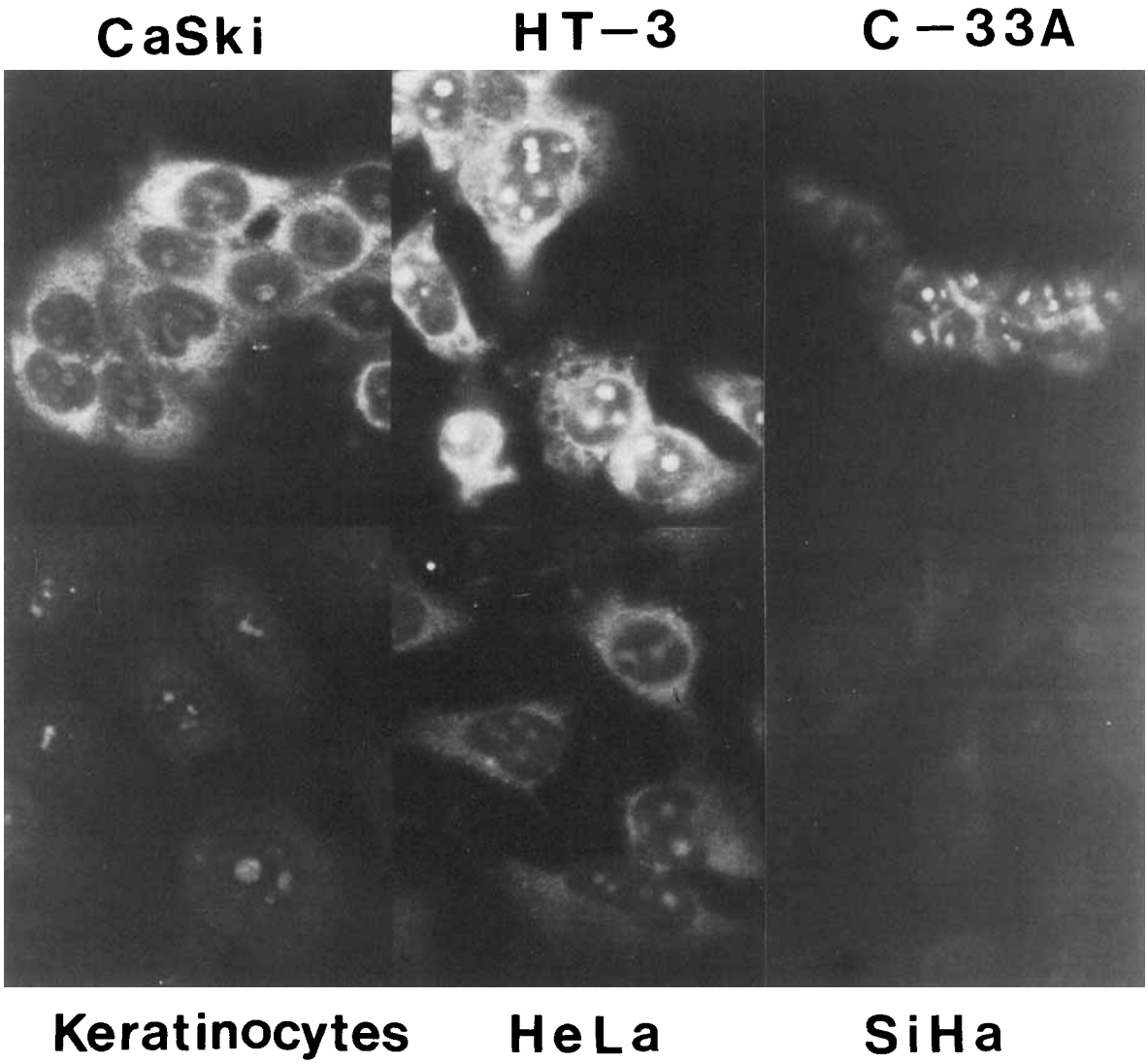

Fig. 3. Distribution of bcl-2 protein in cervical carcinoma cells assessed by indirect immunofluoresence staining. The cervical carcinoma cell lines CaSki, HeLa, SiHa, C-33A, and HT-3 and primary cultured keratinocytes were fixed and processed for bcl-2 protein detection using a 6c8 hamster anti-human bcl-2 monoclonal antibody (1:20 dilution) and FITC-conjugated goat anti-hamster IgG (1:100 dilution). CaSki, HT-3, C-33A, and HeLa cells showed positive staining for bcl-2. Keratinocytes and SiHa cells were negative for bcl-2 staining. Bar = 20 μm.

staining in CaSki cells obtained with the polyclonal bcl-2 antibody. Preincubation of this antibody with baculovirus expressed bcl-2 protein resulted in loss of bcl-2 staining (Fig. 4B). Preincubation of the polyclonal anti-bcl-2 antibody with an unrelated protein, the EGF receptor (also expressed in a baculovirus system), did not diminish bcl-2 staining in CaSki cells (Fig. 4C). These results demonstrate that the polyclonal anti-bcl-2 antibody reacts specifically with, and detects, bcl-2 protein. Thus, using both immunofluorescence and Western blot analysis, we have documented for the first time the existence of high levels of bcl-2 expression in cervical cancer cells.

Genomic Organization and Transcription Activity of bcl-2

High levels of bcl-2 expression in follicular lymphoma are thought to be a consequence of t(14;18) chromosomal translocation which involves the mbr region of the bcl-2 gene [Graninger et al., 1987]. In some types of cervical cancer, HPV DNA integration occurs at a high frequency and could potentially cause rearrangement of cellular DNA sequences [Zur Hausen,

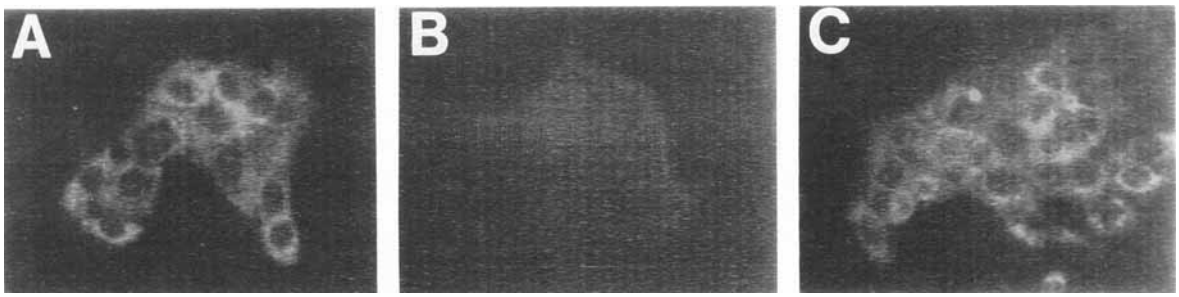

Fig. 4. Specificity of polyclonal anti-human bcl-2 antibody. **A:** CaSki cells were stained with a 1:200 dilution of rabbit anti-human bcl-2 polyclonal antibody followed by rhodamine goat anti-rabbit IgG (1:160 dilution). **B:** Preincubation of anti-human bcl-2 antibody with baculovirus expressed bcl-2 protein resulted in loss of bcl-2 protein staining in cells. **C:** Preincubation of anti-human bcl-2 antibody with an unrelated protein (EGF receptor), also expressed in a baculovirus system, had no effect on bcl-2 staining in CaSki cells.

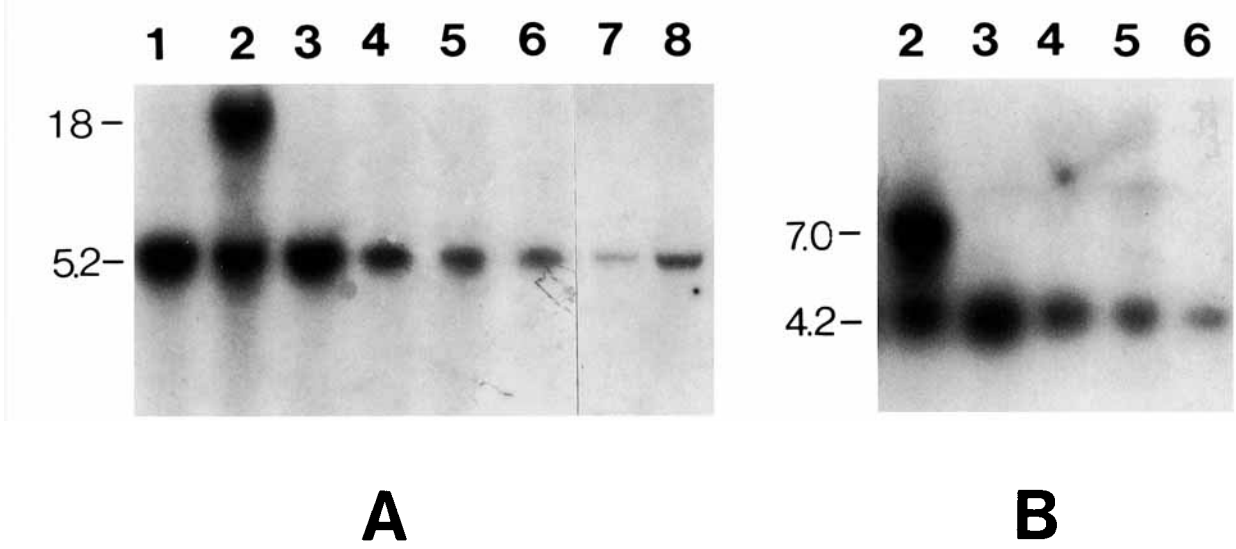

Fig. 5. Southern blot analysis of bcl-2 gene arrangement in cervical carcinoma cells. Ten micrograms of DNAs from placenta (lane 1), SU-DHL-4 lymphoma cells (lane 2), HeLa (lane 3), CaSki (lane 4), HT-3 (lane 5), keratinocytes (lane 6), SiHa (lane 7), and C-33A (lane 8) were digested with EcoR I (**A**) or Hind III (**B**), separated on a 1% agarose gel, and hybridized with a α^{32} P-dCTP labeled bcl-2 probe. Size markers (in Kb) are given at left of the panels.

1991; Howley, 1991]. To determine whether chromosomal translocation or other genomic rearrangement might underlie increased bcl-2 expression in cervical carcinoma cells, we performed Southern blot analysis using a 2.7 Kb bcl-2 DNA probe (which included the mbr region where the translocation is known to occur in follicular lymphoma). In the normal bcl-2 gene, restriction digest with EcoR I yields a 5.2 Kb fragment while digestion with Hind III yields a 4.2 Kb fragment [Tsujimoto and Croce, 1986]. In the follicular lymphoma (SU-DHL-4) cell line which contains the t(14,18) translocation, digestion with EcoR I yields an extra fragment of 18 Kb, while digestion with Hind III results in an extra fragment of 7 Kb. These extra bands are the result of bcl-2 rearrangement occurring at translocated allele. When the DNAs from cervical carcinoma cell lines and keratinocytes were cleaved with either EcoR I or Hind III, only the 5.2 Kb or 4.2 Kb bands were present, respectively (Fig. 5). This finding suggests that the cervical cancer cell lines and keratinocytes do not contain the t(14;18) translocation or other remarkable rearrangement.

To further determine whether bcl-2 gene rearrangement could underlie the increased expression observed in cervical carcinoma cells, we used PCR and primers that specifically spanned the mbr region of either normal or t(14;18)

translocated bcl-2 gene, followed by Southern blot hybridization using an internal oligonucleotide probe, MBR-2, for detection. A normal 202 bp bcl-2 mbr band was present in all cells examined (keratinocytes, all cervical carcinoma cell lines, and the SU-DHL-6 follicular lymphoma cell line; [Fig. 6A]). However, only the SU-DHL-6 cells contained a band of amplified bcl-2/IgH (Fig. 6B). The results from both Southern hybridization and PCR indicate that cervical carcinoma cells, regardless of the presence or absence of integrated HPV DNA, do not have a translocation/rearrangement at bcl-2 mbr region similar to the follicular lymphomas. However, the presence of point mutations or small genomic rearrangement of bcl-2 cannot be ruled out by these analyses.

We next examined the transcription of bcl-2 in cervical cancer cells and keratinocytes. It has been previously shown that bcl-2 transcription is present in the majority of cell lines derived from lymphoid malignancies, but not in HeLa cells [Cleary et al., 1986]. Northern blot analysis was performed using a Raji cell line as a positive control for bcl-2 transcription (Fig. 7). Raji cells contained high levels of 8.5 and 5.5 Kb bands for bcl-2-a and a 3.5 Kb band for bcl-2-b transcript using either total RNA or mRNA preparations. In comparison to Raji cells, relatively low levels of bcl-2-a transcript was found in HeLa, CaSki, and C-33A cells (total RNA, Fig. 7A). Transcript for bcl-2 b was visualized in HeLa cells using mRNA (Fig. 7B, lane 2) and C-33A total RNA (Fig. 7A, lane 5) preparations. As the signals obtained using Northern blot analysis were weak and difficult to interpret, we used RT-PCR to amplify bcl-2 mRNA. The same amount of total RNA from each cell line was used for cDNA synthesis and was detected with a specific bcl-2 oligonucleotide probe. Our ability to detect bcl-2 transcription was dramatically enhanced. With the exception of SiHa cells, all of the cervical cancer cell lines contained bcl-2 transcript which varied in amounts (Fig. 8). These data indicate a strong correlation between bcl-2 transcriptional activity and bcl-2 protein expression in the cervical cancer cells.

DISCUSSION

Numerous studies support the concept that high risk types of HPV are causally involved in pathogenesis of cervical cancer. These "high risk" HPVs have been found in 84% of cervical carcinomas [Riou et al., 1990], generally integrated into the host genome [Zur Hausen, 1991]. The high risk HPV E6 and E7 proteins have

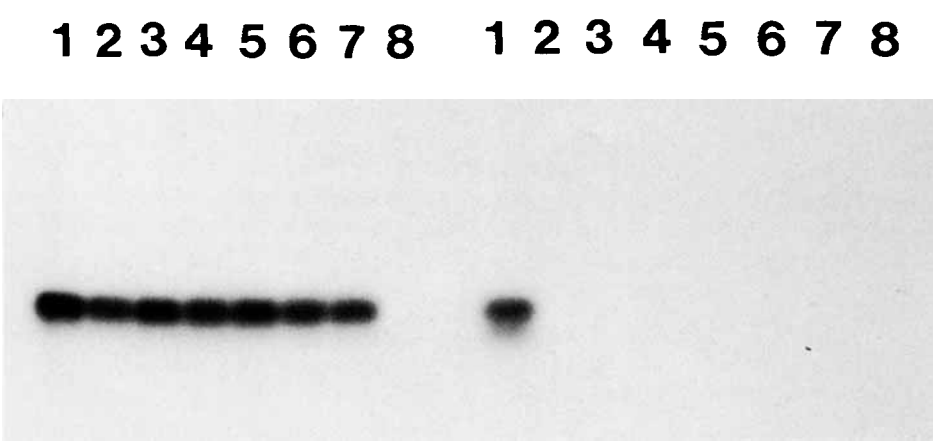

Fig. 6. Examination of bcl-2 mbr gene rearrangement in cervical carcinoma cells by PCR. Genomic DNAs (500 ng) were used in PCR amplification for normal, non-rearranged bcl-2 (mbr) allele with MBR-1 and MBR-3 primers (**A**; see Fig. 1), and for the bcl-2 (mbr)/IGH (JH) fusion allele with MBR-1 and JH-1 primers (**B**; see Fig. 1). Ten microliters of each PCR product was electrophoretically separated on 2% agarose gels and subsequently transferred to nylon membrane. Southern hybridization was performed with α^{32}P-dCTP labeled MBR-2 oligonucleotide. The amplified band is located at 202 bp, shown by the arrow. Lanes: (1) SU-DHL-6 lymphoma cells, containing a bcl-2/IGH translocation allele; (2) HeLa; (3) CaSki; (4) SiHa; (5) C-33A; (6) HT-3; (7) keratinocytes; (8) negative control (no template DNA). Only the SU-DHL-6 lymphoma cells demonstrated positive amplification for bcl-2 (mbr)/IgH (JH) fusion allele.

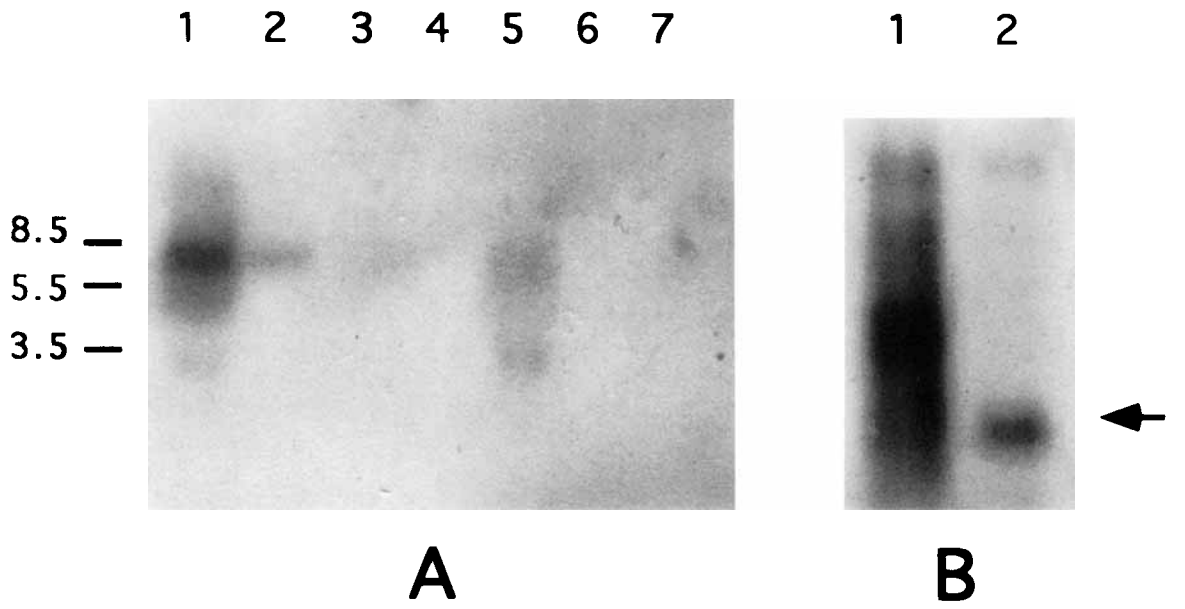

Fig. 7. Northern blot analysis of bcl-2 transcription. Twenty microliters of total RNA (**A**) and 1 μg of mRNA (**B**) were separated on a 1% agarose gel and hybridized with an α^{32}P-dCTP labeled bcl-2 probe. Lanes: (1) Raji, (2) HeLa; (3) CaSki; (4) SiHa; (5) C-33A; (6) HT-3; (7) keratinocytes. Molecular size (Kb) of RNA is shown at the left of the panel. The arrow indicates a 3 Kb bcl-2-b transcript in HeLa cells.

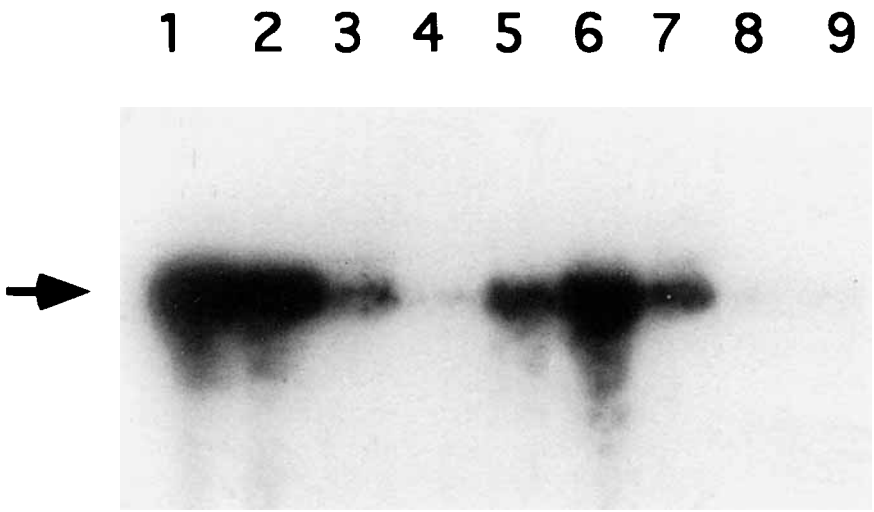

Fig. 8. Detection of bcl-2 transcription with RT-PCR. One microgram of DNase treated RNA was reverse transcribed and then amplified by PCR. The amplified cDNA samples were electrophoretically separated on 2% agarose gel, transferred to nitrocellulose membrane, and hybridized with a α^{32}P-dCTP labeled bcl-2 (DA) oligonucleotide. The amplified transcription bands, located at 330 bp, are shown by the arrow. Lanes: (1) Raji; (2) HeLa; (3) CaSki; (4) SiHa; (5) C-33A; (6) HT-3; (7) keratinocytes; (8) negative control: DNase treated RNA derived from keratinocytes, in which RT was not added during the process of cDNA synthesis before PCR; (9) negative control: a PCR reaction mixture without template.

been suggested to contribute to cervical carcinogenesis as they can cooperatively or separately immortalize human foreskin, cervical keratinocytes, and breast epithelial cells [Münger et al., 1989; Hawley-Nelson et al., 1989; Band et al., 1990]. One of the mechanisms which may be employed by the E6/E7 proteins is inactivation of p53 or RB, respectively, leading to loss of growth (tumor) suppressor activity [Werness et al., 1990; Dyson et al., 1989]. This hypothesis is supported by our recent demonstration that endogenous p53 and E6 proteins associate in the cytoplasm of intact cervical carcinoma cells [Liang et al., 1993], *preventing p53 accumulation*

in the nucleus of cells where it exerts its biological activity. However, E6/E7 expression alone is not sufficient for transformation which requires cooperation with an activated ras oncogene [Dürst et al., 1989].

Although inactivation of p53 is a common event in human cancer [Vogelstein, 1990], the actual biological consequences of its inactivation are only now beginning to become clear. p53 functions in genomic surveillance. Cells devoid of p53 or expressing mutant p53 cannot arrest in the G1 phase of cell cycle or undergo p53-dependent apoptosis in response to DNA damage, leading to genomic instability [Yonish et al., 1991; Demers et al., 1994; Livingstone et al., 1992; Kessis et al., 1993; McCarthy et al., 1994]. p53's ability to negatively regulate cell growth appears dependent on its transcriptional activity. Several genes which are transcriptionally activated by p53 have been identified [Zambetti and Levine, 1993]. One of these is a 21 kD protein (WAF 1 or Cip1), which binds to Cdks and inhibits tumor cell growth [El-Deiry et al., 1993; Harper et al., 1993]. p53 has also been shown to suppress the transcriptional activity associated with the promoters of human c-fos, c-jun, IL-6, MDR-1, and ect [Ginsberg et al., 1991; Santhanam et al., 1991; Chin et al., 1992].

Recently, bcl-2 has emerged as a unique oncogene whose expression permits prolonged cell lifespan [Reed, 1994]. In the adult, bcl-2 is mainly expressed by immature cell populations, such as bone marrow progenitor cells and epithelial precursors [Hockenbery et al., 1991]. Transgenic mice that overexpress bcl-2 display extended survival of B cells, while reduction of bcl-2 expression with bcl-2 antisense oligodeoxynucleotide treatment accelerated the rate of cell death [McDonnell et al., 1989; Reed et al., 1990a]. Bcl-2 deficient mice display retarded growth, fulminant lymphoid apoptosis, and polycystic kidneys [Veis et al., 1993]. Bcl-2 expression is also widespread in embryonic tissues, where it is thought to be important in the accelerated growth required of newly forming tissues and organs [Lebrun et al., 1993]. Over-expression of bcl-2 has been observed to block apoptosis induced by tumor necrosis factor, adenovirus E1A, c-myc, heat shock, chemotherapy, and p53 [Lotem and Sachs, 1993; Hennet et al., 1993; Rao et al., 1992; Bissonnette et al., 1992]. That overexpression of bcl-2 is associated with an inhibition of apoptosis induced by p53, suggests that these two proteins may interact to regulate apoptosis. Like the high risk HPVs, bcl-2 can cooperate with the ras oncogenes to transform rodent fibroblasts rendering these cells tumorgenic in animals [Reed et al., 1990b]. In this study, we found increased levels of bcl-2 expression in 4 of 5 cervical carcinoma cell lines compared to normal keratinocytes. To the best of our knowledge, this is the first report of increased bcl-2 expression in cervical carcinoma cells. These data suggest that overexpression of bcl-2 may contribute to the development of cancers (i.e., cervical cancer) other than B cell lymphoma.

The mechanism(s) responsible for up-regulation of bcl-2 expression in cervical carcinoma cells are unknown. Overexpression of bcl-2 was initially described in B-cell malignancies with a t(14;18) chromosomal translocation. This translocation juxtaposes the bcl-2 gene at 18q21 and IgH locus at 14q32, resulting in constitutive expression of bcl-2. Therefore, we examined cervical carcinoma cells for any evidence of this same translocation or other rearrangement in the bcl-2 gene; no evidence for a t(14;18) chromosomal translocation or any other rearrangement of the bcl-2 gene was found. These findings are in agreement with other studies which document high levels of bcl-2 expression and aberrent patterns of bcl-2 protein production in a variety of solid tumors (e.g., adenocarcinomas of the prostate, squamous carcinomas of the lung and nasopharyngeal carcinomas) in the absence of any evidence of gross alterations in bcl-2 gene structure.

It has been reported that EBV infection can enhance the survival of latently infected B cells, and this enhanced survival correlates with overexpression of bcl-2 protein [Cleary et al., 1986; Henderson et al., 1993]. Upon further examination, sequence analysis showed that the open reading frame encoding EBV BHRF1 protein was 25% homologous with that of the bcl-2 gene and that this homology was sufficient to cause stimulation of bcl-2 expression when EBV BHRF 1 expression was high. As some of the cervical carcinoma cells we examined were infected with HPV, a similar homology between the ORF of HPV and bcl-2 could account for the increased bcl-2 expression we observed. Therefore, we compared the sequence of bcl-2 cDNA with that of HPV 16 and 18 DNA by computer-generated alignment using the MacVector program version 4.0 from IBI/Eastman Kodak (New Haven, CT). No significant homologies were found be-

tween bcl-2 and HPV 16/18, suggesting that HPV may not directly up-regulate bcl-2 expression by the same mechanism as EBV. This result is consistent with our findings of increased bcl-2 expression in HT-3 and C-33A cervical carcinoma cells, which are not HPV infected but still display increased bcl-2 expression.

The one consistent phenomenon of our study is the finding that high levels of bcl-2 expression occurred in cells lacking functional p53. In the cervical carcinoma cells examined, p53 is inactivated via either HPV E6 protein binding (HeLa and CaSki) or p53 mutation (C-33A and HT-3) [Scheffner et al., 1991; Srivastava et al., 1992]. Normal keratinocytes, which contain wild type p53, did not demonstrate bcl-2 expression (no protein expression could be found by either immunoblotting or immunofluorescence). Although our data did indicate bcl-2 transcription by RT-PCR in keratinocytes, the amount of bcl-2 transcription was relatively low as indicated by the fact that it was undetectable by Northern blot analysis. The undetectable bcl-2 protein may be due to a short half life of bcl-2 mRNA or a negative post-transcriptional regulatory mechanism in normal keratinocytes.

Recently, high levels of bcl-2 expression have been reported in p53-negative tumors [Silvestrini et al., 1994] and in p53 deficient mice (Miyashita et al., 1994). These findings in conjunction with the results presented here raise the hypothesis that bcl-2 may be transcriptionally repressed by functional p53. Support for such a hypothesis comes from recent findings which indicate that overexpression of p53 induces down-regulation of bcl-2 expression at both the protein and mRNA level [Haldar et al., 1994; Miyashita et al., 1994], although whether this occurs via a direct effect of p53 on the bcl-2 promoter or via an indirect mechanism is not clear. p53 itself can induce apoptosis in response to DNA damage [Yonish et al., 1991; Lowe et al., 1993; Ramqvist et al., 1993], and p53 may exert this activity by keeping the bcl-2 protooncogene "off." However, when p53 function is abrogated, as is the case in HPV-infected cervical carcinoma cells or in cervical carcinoma cells containing mutated p53, the repression of bcl-2 gene by p53 is removed, allowing its expression. Further studies using temperature-sensitive mutant p53 clones transfected into cervical carcinoma cells that possess mutated or wildtype p53 will be necessary to test this hypothesis.

p53 may not be the only regulator controlling bcl-2 activation. The use of p53 deficient mice indicated that with the exception of the prostate, absence of p53 expression did not alter levels of bcl-2 expression [Miyashita et al., 1994]. However, a certain percentage of breast and prostate carcinomas have been found to express high levels of bcl-2 and p53. Our experiments showed that SiHa cervical carcinoma cells (which contain p53 complexed with E6 and therefore supposedly inactive p53) had no bcl-2 expression. Thus, the aberrant state of p53 and bcl-2 protein levels could be independent events, but coordination of their separate biological functions may efficiently block the apoptotic pathway, which may be a very important mechanism in development or progression of cancer. Future studies will examine the interplay between the p53 and bcl-2 proteins.

A caveat that must be kept in mind when undertaking studies such as those described in this paper, is the relevance of findings obtained using tissue culture models of tumors. Findings in cultured cell lines do not necessarily reflect those of primary tumors as has recently been demonstrated in the case of p16. Conversely, studies by Gray and colleagues [1994] examining genomic instability in a variety of breast cancers using comparative genomic hybridization, indicate excellent agreement between chromosomal alterations found in cultured breast cancer cell lines and primary tumors. Studies examining p53 and bcl-2 levels in primary tumors of the cervix are underway and will allow us to address the relevance of the use of cultured cervical tumor cell lines to events which occur in primary tumors.

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