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Effect of dolastatin 10 on human non-Hodgkin's lymphoma cell lines

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It is crucial to incorporate new and more potent antineoplastic agents in treating non-Hodgkin's lymphoma since standard chemotherapy fails to cause a significant increase in the survival rate. A potential chemotherapeutic agent is dolastatin 10; hence, the objective of our study is to investigate the effect of the antiproliferative agent dolastatin 10 on different grades of non-Hodgkin's lymphoma cell lines. All cell lines exposed to dolastatin 10 initiated an apoptosis process. Alteration of oncogenes and their product may direct the entry of the cells into apoptosis, among these oncogenes are *bcl*-2 and c-*myc*. All cell lines tested expressed c-*myc* and *bcl*-2 proteins. However, 24 h after exposing the cell lines to 1 ng/ml dolastatin 10, *bcl*-2 expression was abolished but there was no significant change in c-*myc* protein expression. The contradictory roles of c-*myc* in cell proliferation and death require that other gene(s) products regiment the outcomes of c-*myc* activity on a cell. A possible candidate for such a modifying gene is *bcl*-2, whose product prolongs cell survival and blocks apoptosis. Given the above, dolastatin 10 induction of cell arrest is the initiating signal to downregulate the anti-apoptotic *bcl*-2 and reactivate the apoptotic pathway. The reductions in *bcl*-2 may stabilize the c-*myc* proliferative action and induce apoptosis.

Key words: *bcl*-2, c-*myc*, dolastatin 10, non-Hodgkin's lymphoma.

Introduction

Non-Hodgkin's lymphoma, classified as high, intermediate and low grade, represents a group of malignancies mostly of B lymphocytes arrested at certain stages of the B cell differentiation pathway.[1] Classical chemotherapy cures approximately 40% of the high and intermediate grade lymphomas, but fails to cause a significant increase in the survival rate of the low grade types.[3] Hence, it is crucial to incorporate new and more potent antineoplastic agents in treating these malignancies.[4] A potential chemotherapeutic agent is dolastatin 10, which is isolated from the sea hare *Dolabella auricularia*. It is a cytotoxic antineoplastic compound whose structure was revealed in the late 1980s.[5] Dolastatin 10 inhibits cell proliferation and arrests cells in mitosis by binding to microtubule components, mainly tubulin.[6] Our initial studies with this natural product indicated a high level of growth inhibition in a diffuse large lymphoma cell line (WSU-DLCL2).[4]

The rate of tumor growth is a function of cell proliferation and cell death. In the past most investigators targeted proliferation as a way to control tumor growth.[7] Recently, however, attention has been focused on cell death. Spontaneous or treatment-induced program cell death (apoptosis) is a major mechanism by which tumors shrink.[8,9] The intrinsic inclination of tumor cells to undergo apoptosis as a result of some anti-cancer drug treatment has been shown.[4,10–12] Moreover, engagement of several oncogenes such as *bcl*-2[13] or the tumor suppressor gene, p53,[14] in apoptosis has been suggested. For instance, transgenic mice that overexpress *bcl*-2 accumulate large numbers of small B lymphocytes and in many ways mimic low grade lymphoma.[15]

Chromosomal translocation t(14,18) was reported in follicular lymphoma[13] in which the breakpoint on chromosome 18 is in the 3′ untranslated portion of the *bcl*-2 gene and that on chromosome 14 is in the immunoglobulin heavy chain J region.[13,16] Normal *bcl*-2 is expressed at high levels in certain stages of B cell development.[17,18] Thus, downregulation of *bcl*-2 is a conceptual target for antilymphoma therapy. Another oncogene of interest is c-*myc*. In 1993, Amati *et al.*[19] demonstrated that c-*myc* can control two alternative cellular pathways: cell cycle progression and apoptosis. The critical role of *myc* protein in modulating proliferation and apoptosis and its interaction with other oncogenes needs further studies.

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In this study, we chose three non-Hodgkin's B lymphoma cell lines to investigate the dolastatin 10 effect on cell cycle and oncogene modulation. We report that dolastatin 10 has an antiproliferative effect on all of them. Moreover, it abolished *bcl*-2 protein, but not c-*myc* expression leading to apoptosis. Since dolastatin 10 is more effective in inhibiting tubulin polymerization compared with vincristine,[20] it has a potential for clinical application. The findings presented in this study can be used to guide its further clinical development.

Methods

Cell lines and culture

Three human non-Hodgkin's B lymphoma cell lines available in our cell bank were chosen for this study. These lines represent different types of B cell lymphoma as described by the International Working Formulation[21] as follows: WSU-FSCCL, a low grade, follicular small cleaved cell lymphoma (FSCCL);[22] WSU-NHL, an intermediate grade, follicular large cell lymphoma;[23] and WSU-BL, a high grade, small cell non-cleaved lymphoma (Burkitt's).[24] The cell lines were cultured in RPMI 1640 medium with L-glutamine (Gibco BRL, Gaithersburg, MD) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% (v/v) heat-inactivated fetal calf serum (Hyclone, Logan, UT). Tissue culture flasks then were incubated at 37°C, 5% CO_2 in a humidified incubator.

Treatment with dolastatin 10

Dolastatin 10 was isolated from the sea hare *Dolabella auricularia*.[5] It was dissolved in dimethylsulfoxide at 10^{-3} mg/ml, further diluted with PBS and then was added to the culture cells for a final concentration of 1 ng/ml. This concentration was shown to inhibit proliferation in B cell lymphoma cell lines.[4,25] Furthermore, our pilot study on WSU-BL cells confirmed the former observation. Cells from each line were seeded in 25 cm^2 tissue culture flasks (Falcon Labware, Oxford, CA) at a concentration of 10^6/ml. Incubation of cultures was at 37°C, 5% CO_2 in a humidified incubator.

Cell growth analysis and examination of apoptosis

Cells were seeded in 24-well culture plates at a concentration of 0.3×10^6 viable cells/ml. Untreated (control) and dolastatin 10-treated cultures were set in four replications.

Cell count and viability were obtaind at an interval of 2 h for the first 8 h and then daily using 0.4% trypan blue stain and a hemocytometer. Aliquots from all cultures were cytocentrifuged on 12 mm circle coverslips using a cytospin 2 centrifuge (Shandon Southern Instruments, Sewickley, PA). Cell smears were air dried, stained with tetrachrome for 5 min and analyzed under a light microscope. Five different high power fields were counted for viable, mitotic and apoptotic cells at intervals of 2 h for the first 8 h and then after 24 h. At least 300 cells were evaluated from each treatment and statistically analyzed. Features of apoptosis that were looked for included nuclear chromatin condensation, formation of membrane blebs and apoptotic bodies. Features of cell death (necrosis) included cell swelling, nuclear expansion and gross cytolysis.

DNA fragmentation

DNA fragmentation was conducted as described before.[4] Briefly, human lymphoma cells were cultured for 24 h in the presence of 1 ng/ml of dolastatin 10. A total of 2×10^6 cells were harvested from each treatment, washed in PBS and lysed with 400 μl of lysing buffer [10 mM Tris, pH 7.5, 1 mM EDTA (TE); 0.2% Triton X-100]. DNA was precipitated from the lysate by 0.1 vol. of 5 M sodium acetate and 3 vol. of 95% ethanol. After centrifugation, DNA pellets were air dried and resuspended in 20μl of TE containing 0.1% SDS. Loading buffer was added to the sample at 1:5 (v/v) ratio and the samples were incubated for 10 min at 60°C, followed by agarose gel (0.75%) electrophoresis in Tris borate buffer (45 mM Tris borate, 1 mM EDTA, pH 8.0). DNA was visualized with ethidium bromide under UV light and photographed.

Immunocytochemistry and Western blot analysis for protein detection

As previously described for immunocytochemistry,[26] cells were stained with human anti-c-*myc* (Oncogene Science Uniondale, NY) and human anti-*bcl*-2 (Dako, Carpinteria, CA) for detection of c-*myc* and *bcl*-2 proteins, respectively. Briefly, cells from appropriate aliquots were cytocentrifuged on 12 mm circle coverslips. They were fixed with 95% ethanol and 5% glacial acetic acid for 10 min at room temperature followed by three washes with 0.1 M PBS. Coverslips were incubated in mixtures of PBS with 1% bovine serum albumin (BSA) for

30 min and then incubated overnight with the primary antibody at the specified concentration. Cells were extensively washed with PBS followed by another wash with a mixture of PBS and BSA. Finally, cells were incubated for 2 h with the secondary antibody conjugated to alkaline phosphatase. A subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) produced a blue precipitate which visualized the target protein. Western blot analysis to detect the same proteins was performed using a modified method of Li and Chen.[27] Briefly, the cells obtained from untreated and dolastatin 10-treated cultures were lysed in 100 μl/10^6 cells of cold lysis solution (10 mmol/l Tris–HCl, pH 7.6; containing 1% Triton X-100; 10% glycerol; 0.2 mM sodium orthovandate; 150 mmol/l NaCl; 5 mmol/EDTA; and 2 mmol/l phenylmethylsulfonyl fluoride; 10 μg/ml pepstatin; and 10 μg/ml aprotinin) and incubated on ice for 30 min. Insoluble debris were removed by centrifugation at 4°C for 10 min at 13 000 r.p.m. Total protein content of the lysates was determined by using a protein assay kit provided by Sigma (St Louis, MO). Total cell lysates were boiled in an equal volume of 2 × SDS sample buffer with 2-mercaptoethanol for 5 min. The samples were subjected to electrophoresis on 8 × 10 cm, 8% gradient one-dimensional SDS–PAGE. After electrophoresis, proteins were transferred from the gel to a 0.2 mm nitrocellulose filter (Schleicher & Schuell, Keene, NH) at 14 V overnight at 4°C. Non-specific binding sites on the filter were blocked by incubating the nitrocellulose filter in blocking buffer (5% skim milk in PBS) for 1 h at room temperature. The blots were washed in PBS containing 0.05% Tween 20 and incubated for 1–2 h at room temperature with primary mouse anti-c-*myc* (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-*bcl*-2 (Oncogene Science) antibodies. After the removal of primary antibodies with extensive washes, an alkaline phosphatase-conjugated secondary goat anti-mouse IgG (Sigma) was employed. Immunoblots were developed as described above using the enzyme-catalyzed color reaction.

Results

Cell growth and cell cycle changes

The effects of 1 ng/ml dolastatin 10 on the growth of three grades of non-Hodgkin's B lymphoma cell lines are shown in Figure 1. Dolastatin 10 caused a significant growth arrest in cell lines studied; however, the effect was more pronounced on low (WSU-FSCCL) and intermediate (WSU-NHL) grade B lymphomas after 3 days of treatment. No signs of recovery were observed thereafter.

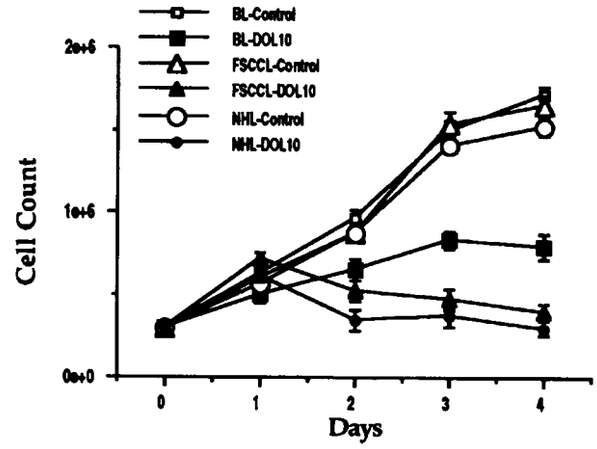

Figure 1. Growth curves of non-Hodgkin's lymphoma cell lines WSU-BL (BL), WSU-FSCCL (FSCCL) and WSU-NHL (NHL) after 1 ng/ml dolastatin 10 treatment (DOL-10) or dilutent (Control).

Human non-Hodgkin's B lymphoma cell lines were assessed morphologically for viability, mitosis, apoptosis and death (Figures 2 and 3). All the non-Hodgkin's B lymphoma cell lines treated with 1 ng/ml dolastatin 10 showed characteristic features of apoptosis, mitosis and cell death (Figure 2). At 24 h after dolastatin 10 treatment, mitotic arrest was seen in 32 ± 5% of WSU-BL, 19 ± 6% of WSU-NHL and 17 ± 3% of WSU-FSCCL. Simulataneously, apoptosis was induced in 30 ± 5, 25 ± 7 and 15 ± 4%, respectively, as compared with the control which showed no apoptosis (Figure 3). Dolastatin 10 caused death in 6 ± 2% of the WSU-BL, 8 ± 3% of WSU-NHL and 7 ± 2% of WSU-FSCCL (data not shown).

To confirm that cell death was induced by an apoptotic pathway, electrophoresis anlaysis was conducted on dolastatin 10-treated cells. A classical laddering pattern, resulting from the generation of internucleosomal DNA fragmentation, was seen in all cell lines tested after dolastatin 10 treatment, indicating that these cells had gone through an apoptotic pathway (Figure 4).

c-*myc* and *bcl*-2 expression

To determine whether modulation of c-*myc* and *bcl*-2 is associated with the development of apoptosis on non-Hodgkin's B lymphoma cell lines, c-*myc* and *bcl*-2 oncoprotein expression was examined

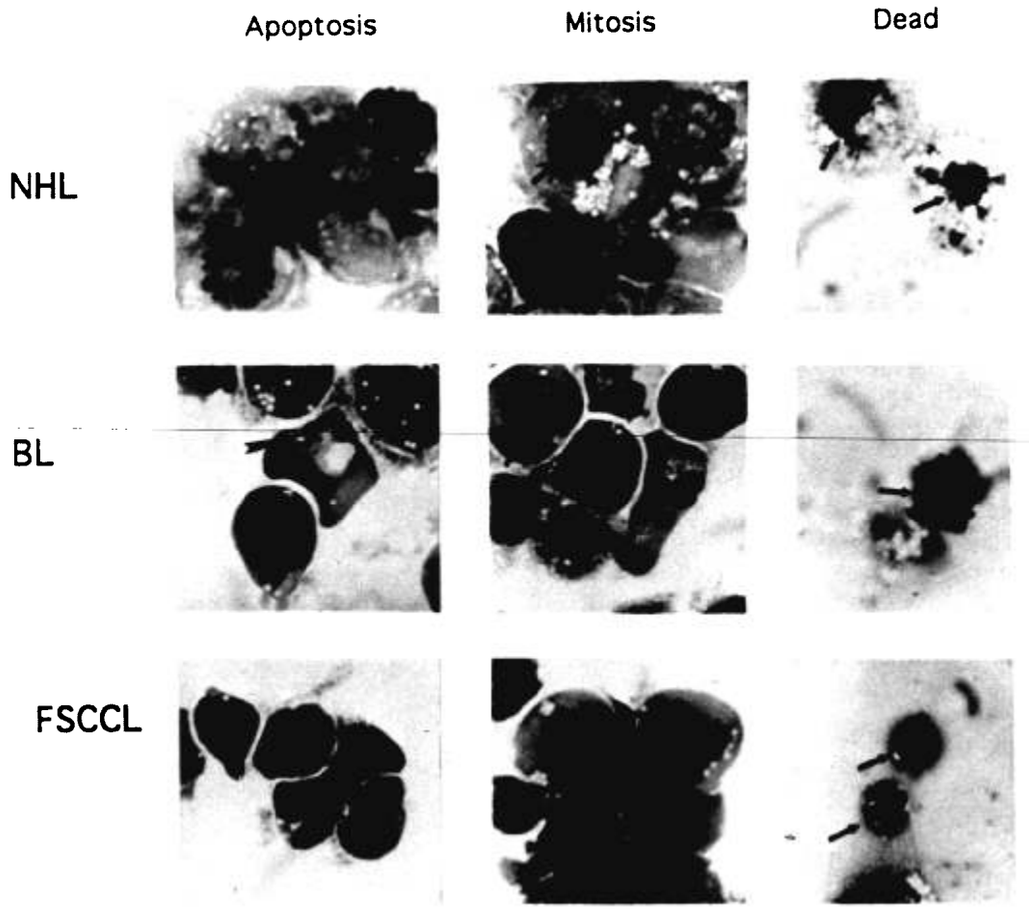

Figure 2. Representative photograph showing the characteristic features of apoptosis, mitosis and dead cells in non-Hodgkin's lymphoma cell lines WSU-BL, WSU-NHL and WSU-FSCCL after 1 ng/ml dolastatin 10 treatment. Notice the apoptotic bodies (arrow), which are a main feature of apoptic cells, mitotic spindle for cells in mitosis (arrow) and necrotic cells (arrow).

utilizing c-*myc* and *bcl*-2 mAbs. All untreated (control) cell lines strongly expressed c-*myc* and *bcl*-2 using Western blotting (Figure 5) or immunocytochemistry (Figure 6). In all cell lines tested, however, following treatment with dolastatin 10, the expression of *bcl*-2 protein was abolished, but there was no significant change in c-*myc* expression. Hence, dolastatin 10 was not effective in modulating c-*myc* expression but it has a dramatic effect on *bcl*-2.

Discussion

Until two decades ago, the advanced stage non-Hodgkin's lymphoma was a uniformly fatal disease. However, with the introduction of combination chemotherapy, i.e. cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP), 30–40% of the intermediate and high grade lymphomas are cured.[3] Since then, several combinations of standard chemotherapy agents have been developed in an attempt to improve the cure rate.[28,29] Unfortunately none of these regimens has proven to be superior to the original CHOP in randomized clinical trials.[29] The low grade lymphoma (which constitute 40% of all lymphomas) has remained incurable, although bone marrow transplantation may alter this outlook for selected patients.[30] Hence, there is a clear need for new compound(s) and a better understanding of the proliferation and survival signaling pathways within lymphoma cells.[4–6] Dolastatin 10 is the first compound of marine origin with capacity to arrest dividing cells in G_2/M.[6,31,32] This agent is more

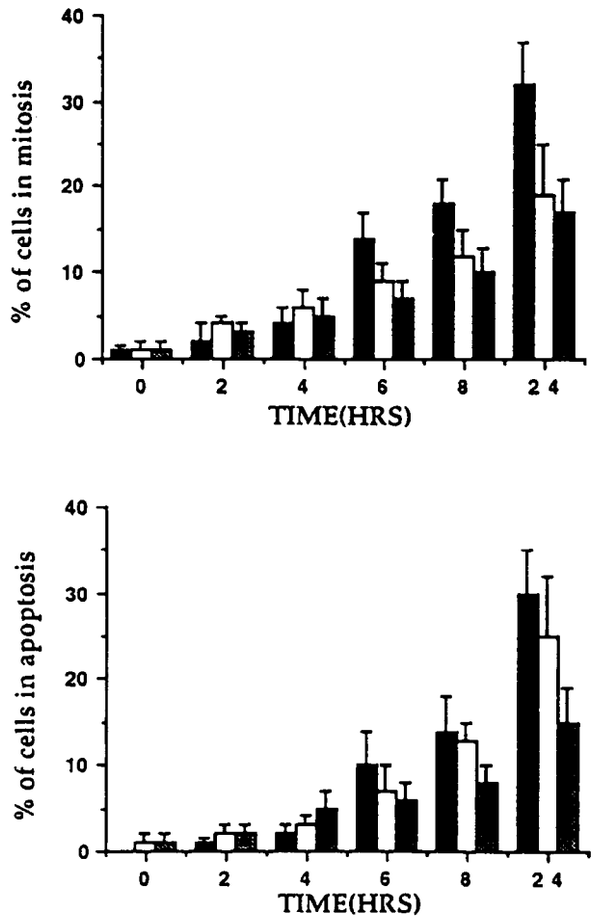

Figure 3. Bars represent percentage of cells in mitosis or apoptosis relative to total number of cells. Three replicates were used for each treatment and five different high power fields were counted for mitotic and apoptosis cells at intervals of 2 h for the first 8 h and then after 24 h. At least 300 cells were evaluated from each treatment and statistically analyzed. ■, WSU-BL; □, WSU-NHL; ▨, WSU-FSCCL.

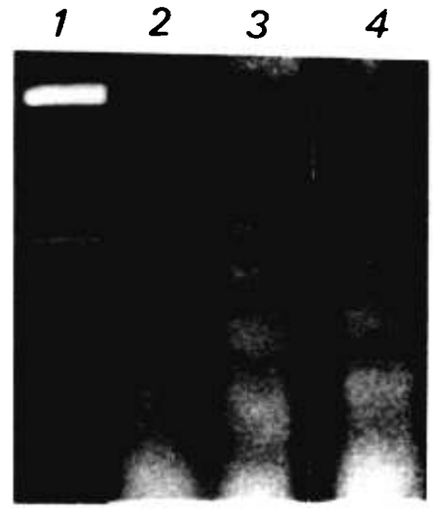

Figure 4. Nucleosomal DNA fragmentation in dolastatin 10-treated non-Hodgkin's lymphoma cell lines WSU-BL, WSU-NHL and WSU-FSCCL 1 day after dolastatin treatment. Lane 1, DNA mass ladder provided by Gibco/BRL (200–800 bp); 2, WSU-FSCCL; 3, WSU-BL; 4, WSU-NHL.

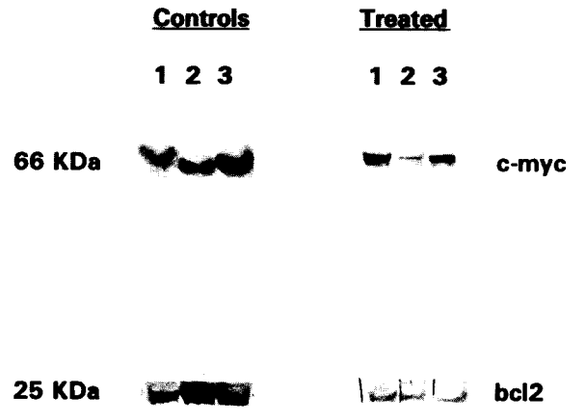

Figure 5. Western blots for detection of c-*myc* (top) or *bcl*-2 (bottom) protein expression in WSU-FSCCL (1), WSU-BL (2) of WSU-NHL (3). Dolastatin 10 suppresses expression of *bcl*-2 product but fails to change expression of c-*myc*.

effective in inhibiting tubulin polymerization than the vinca alkaloid vincristine,[6,20] which is widely used in the treatment of a variety of human cancers including lymphoma.[33] These observations formed the rationale for our testing of dolastatin 10 in a group of malignant lymphoma lines.

Our results confirm those of Beckwith *et al.*[25] that dolastatin 10 has a growth inhibitory effect on lymphoma cell lines. Similar to their findings, we also demonstrated arrest in mitosis and induction of apoptosis as the major pathways of cell cycle arrest and death. In order to further investigate the mechanism of dolastatin-induced apoptosis, we chose to evaluate *bcl*-2 and c-*myc* expression. *bcl*-2 is activated/dysregulated in a variety of B lymphoid tumors,[34] and is known to inhibit apoptosis.[35] The c-*myc* oncogene, usually implicated in cell proliferation, also plays a central role in some forms of apoptosis.[36] It has been demonstrated in Chinese hamster ovary cells[37] and Rat-1 fibroblasts[38] that *bcl*-2 can abrogate c-*myc*-induced apoptosis. The opposing roles of *myc* in cell growth and death seem to be dependent on a 'second signal'. The expression of *bcl*-2, which inhibits apoptosis, is considered a 'survival signal', which allows more *myc* to drive the cell through the cell cycle. Examples of other survival signals are growth factors like IL-3.[36] Without a survival signal, c-*myc* induces apoptosis. In our study of three lymphoma cell lines expressing

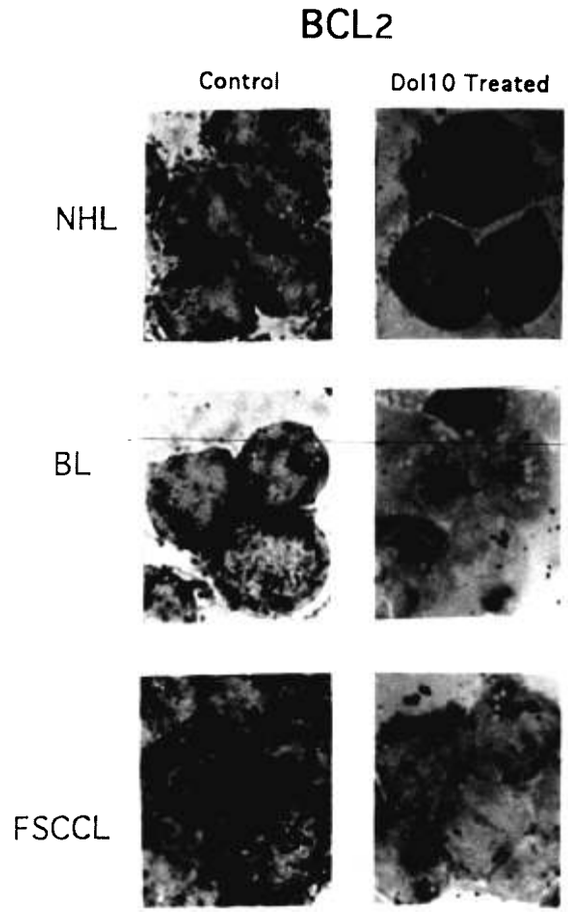

Figure 6. Immunocytochemistry of *bcl*-2 protein expression for untreated and dolastatin 10-treated non-Hodgkin's lymphoma cell lines WSU-BL, WSU-NHL and WSU-FSCCL. Notice that *bcl*-2 expression was suppressed in dolastatin 10-treated cells. There was no significant change in c-*myc* staining (not shown).

both c-*myc* and *bcl*-2 proteins (Figure 5), dolastatin 10 removes the 'survival signal' by abolishing *bcl*-2 (Figures 5, bottom, and 6). According to the 'two signal' model, c-*myc*, unaffected by dolastatin 10, induces apoptosis. These observations can facilitate a rationale to develop new therapeutic combinations for lymphoma.

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