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Bone biopsy derived marrow stromal elements rescue chronic lymphocytic leukemia B-cells from spontaneous and drug induced cell death and facilitates an “angiogenic switch”

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Abstract

A novel bone biopsy technique was used to generate a robust stromal cell system to study how stroma modulates CLL B-cell apoptosis and how the leukemic cell–stromal interaction influences secretion of vascular factors. Marrow stromal elements (MSE) rescued CLL B-cells from both spontaneous and drug induced apoptosis, partly due to soluble factors. When CLL B-cells were added to the MSE cultures, a dramatic increase in the secretion of basic fibroblast growth factor and decrease in the secretion of thrombospondin was observed. These results indicate the interaction between CLL B-cells and marrow stromal elements regulates angiogenic switching and may be linked to disease progression.

Keywords

Marrow; Stromal cells; CLL; VEGF; Apoptosis

1. Introduction

CLL B-cells are predominantly characterized by their ability to resist apoptosis [1,2], but this biologic feature is lost when CLL B-cell are removed from the host. These facts suggest interactions between the CLL B-cell and its *in vivo* environment can modulate the CLL B-cell apoptotic status so that the CLL B-cell is better able to survive. Multiple cytokine and cell-cell interactions in various tissue sites are likely involved in complex networks that facilitate this apoptotic resistance. A number of cytokines including VEGF, IL-2, IL-4, IL-10 and Interferon- α and - γ are known to enhance CLL B-cell survival [3–7]. There is also substantial evidence that physical contact with stromal elements can facilitate CLL B-cell apoptosis resistance [8–10]. These interactions are not mutually exclusive where cell-cell interactions can lead to secretion of cytokines which can then be concentrated by glycosaminoglycans in the extracellular matrix to provide survival signals to leukemic cells. Dissecting the kinetics and coordination of this complex array of interactions and their relative importance in generating enhanced CLL B-cell survival is necessary if these pathways are to be targeted with therapeutic intent.

The earliest stages of B-CLL are characterized by bone marrow infiltration and circulating leukemic CLL B-cells implying the marrow microenvironment is a critical site of nurturing in

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the disease process. Direct physical contact between bone marrow stromal cells and the leukemic B-cell using $\beta 1$ and $\beta 2$ integrins [11] extends leukemic cell survival [11–13]. Since this binding rescue CLL cells from apoptosis, it is a potential mechanism for the preferential *in vivo* accumulation and survival of CLL cells within the marrow. Other studies have demonstrated the expression of $\alpha 4/\beta 1$ integrin allows CLL B-cells to interact with the activated endothelium to increase expression of vascular-cell adhesion molecule (VCAM-1) in bone marrow and secondary lymphoid organs [14]. Recently, it has also been shown that CD100 (found on CLL B-cells) and its high-affinity receptor Plexin-B1 (expressed by BM stromal cells) can result in cell–cell interactions which generate both increased proliferative activity and an extended lifespan for CLL B-cells [12].

We and others have also demonstrated that CLL marrow sites contain abnormal vascular elements that appear to be related to disease stage and are predictive of poor clinical outcome when seen in early stage disease [15–17]. This neovascularization may be related to the ability of CLL B-cells to secrete VEGF and other angiogenic factors which, in turn, induce marrow vascularity. Since we also know that CLL B cells can secrete anti angiogenic factors such as thrombospondin-1 (TSP-1), the possibility of an angiogenic switch is raised in CLL. Most malignant cells cannot grow to a clinically detectable tumor mass in the absence of blood vessels [18]. Thus, in order for a tumor to become large it has to switch on an angiogenic phenotype to support their growth. An angiogenic phenotype switch is most simply an imbalanced expression of angiogenic factors (i.e., VEGF) and anti-angiogenesis inhibitors (i.e., TSP-1) [19]. These biologic observations underscore the significance of the continuous, intimate, and potentially bi-directional interaction between the CLL B-cells and their microenvironment. Based on these observations, many groups are attempting to more clearly define the nature of CLL B-cell interaction with marrow elements and how it relates to leukemic cell apoptotic resistance.

In the present work, we utilize a novel technique to establish long-term marrow cultures from CLL patients and normal donors using bone biopsies. This source of primary stromal cells differs from previous work using stromal cell lines or mononuclear cells from bone marrow aspirates as a source of stromal cells [11,20,21]. The main feature of this long-term bone marrow culture is the initial establishment of an adherent cellular environment containing four major stromal cell types: epithelial fibroblast like cells; endothelial cells; phagocytic cells; and large adipocytes [22]. Using this approach, CLL marrow cultures could be maintained *in vitro* for >12 months and were used to evaluate functional interactions between CLL B-cells and the marrow microenvironment that influence both spontaneous and drug induced apoptosis. Finally, we demonstrate that interactions between CLL B-cells and primary bone marrow stromal cells lead to a simultaneous increase in marrow secretion of specific pro-angiogenic cytokines and decrease in anti-angiogenic cytokines suggesting that CLL B-cell-marrow stromal interactions may facilitate an angiogenic switch that favors leukemic cell survival.

2. Materials and methods

2.1. Patient population and blood specimens

Blood and bone marrow biopsies were obtained from CLL patients who had provided written informed consent under a protocol approved by the Mayo Clinic Institutional Review Board according to the regulations of the Declaration of Helsinki. All CLL patients had a confirmed diagnosis using the NCI Working Group definition [23]. Patients in this cohort were from all Rai stages and had not been treated for at least 4 weeks prior to blood processing. Patient age ranged from 44 to 81 years (median, 62 years). CLL cells were isolated from heparinized venous blood by density gradient centrifugation. Purified lymphocytes from CLL patients were

either used immediately (≤ 48 h) for the laboratory studies described below or suspended in RPMI 1640/20% fetal calf serum/10% DMSO and stored at -80°C until used.

2.2. Bone biopsy derived stromal cell cultures

Primary bone biopsy stromal cell cultures were established using a modified version of a previous published technique [22]. In brief, fresh bone marrow core biopsy samples were obtained from CLL patients ($n = 25$), placed in 15-ml tubes containing 10 ml of RPMI 1640, and picked up within 1 h of collection. Control bone biopsies, obtained from waste bone material of patients undergoing hip replacement, were handled in identical fashion. Long-term bone marrow cultures from these samples were then established. Prior to culture, the biopsy core was placed in a culture dish filled with MyeloCult media (05150, StemCell Technologies, Vancouver, Canada) under sterile conditions. Using a scalpel and a forceps, the biopsy piece was broken up into tiny fragments which were distributed in five 35×10 mm culture dishes. To each dish, 2-ml culture medium MyeloCult was added. In addition, 10^{-6}M hydrocortisone, 100 U/ml penicillin and 100- $\mu\text{g}/\text{ml}$ streptomycin were added in the medium to avoid bacterial contamination. The culture medium contained neither growth factors nor cytokines. One culture dish filled with RPMI 1640 was included to maintain internal humidity. All culture dishes were then placed in a single, larger 150×50 -mm dish. Cultures were incubated at 37°C in humidified atmosphere containing 5% CO_2 and fed every week with MyeloCult medium by half medium change. We studied the *in vitro* growth patterns of primary and secondary CLL BM cultures (see below). When marrow stromal cell elements (MSE) cultures were 80–100% confluent (typically between 8 and 10 weeks), cells and culture supernatant were used for the apoptosis/cell death studies or the angiogenesis studies detailed below.

2.3. Establishment of secondary cultures from CLL bone biopsy derived MSE

After reaching confluence, the adherent cells from selected cultures ($n = 7$) were treated with 0.05% trypsin (GIBCO) and transferred to six-well plates and/or 100 mm plates depending on the amount of cells (first passage). Adherent cells were suspended in alpha MEM with 20% fetal bovine serum (BioSource), 2.0 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Neither growth factors nor cytokines were added to the culture medium. These cells were maintained until confluence and were again trypsinized and resuspended in T-75 flask (second passage). At the end of second passage when the MSE reached confluence, they were trypsinized and cryopreserved for future use or placed in culture to be used in experiments of CLL B-cell apoptosis/cell death or the angiogenesis studies described below.

2.4. Apoptosis assays

In order to evaluate the effect of various stromal cell types or culture conditions on CLL B-cell apoptosis, we cultured primary CLL B-cells with primary bone biopsy derived MSE (both CLL and normal donor derived), stromal cell lines (HS-5 [24]), and fibronectin coated plates. For these experiments, the stromal cells were typically placed in a six-well plate in serum-free alpha MEM media until the cells were confluent. Stromal cells were then washed twice with PBS and cultured in serum-free medium (RPMI or Aim V) for 24 h prior to the addition of CLL B-cells. In some experiments we added chlorambucil at various doses for 24–72 h as described below. Leukemic cells were then collected, washed with sterile saline and examined for viability/apoptosis status by staining with annexin V-fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI) as we have previously described [25,26]. At least 5–10,000 events were collected by using CELL-Quest software. In addition to chlorambucil, two drugs known to induce cell death by generating oxidative stress, glucose oxidase and adaphostin, were also tested in this system since we and others have demonstrated the latter two drugs induce death in CLL B-cells [25,27].

2.5. Levels of apoptotic proteins in CLL B-cells

2.5.1. Western blotting—HS-5 human stromal cells or MSE were cultured in DMEM with 10% FCS, 2.0 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin on 100 mm plates. When confluent, stromal cells were washed twice with PBS and cultured in serum-free medium (RPMI or Aim V) for 24 h. CLL B-cells were cultured alone or together with MSE, HS-5 human stromal cells, or fibronectin coated plates for 24–72 h. CLL B-cells were then collected by vigorous pipetting from these co-cultures, washed with PBS, and lysed in lysis buffer. Equal protein was separated on 7.5 and 15% SDS-PAGE, transferred to nitrocellulose membrane and blocked with 10% milk in Tris saline milk (TSM). The membrane was probed with antibodies specific for various apoptotic proteins (i.e., Mcl-1, XIAP, Bcl-2, survivin), washed with PBS/TSM and incubated with HRP-conjugated anti-peroxidase as a secondary antibody for 1 h. Specific bands were developed by chemiluminescence (Amersham). To confirm equal loading of the blots, the membrane was re probed with monoclonal β-actin antibody (Abcam).

2.5.2. Flow cytometry—Samples of CLL B-cells $(0.5\text{--}1.0) \times 10^6$ cells were washed once in PBS, then fixed in Pharmingen Fixative for 20 min and washed with Pharmingen Permeabilization Solution. The cells were incubated for 45 min at 4 °C with 20 µg/ml anti-Bcl-2 monoclonal antibody (Mouse antihuman-Bcl-2, Dako). The same was done for XIAP (Pharmingen Corp., CA), survivin (Novus Biologicals, CO), and Mcl-1 monoclonal antibodies. The cells were washed in the permeablizing solution and incubated for 15 min at 4 °C with antimouse IgG-FITC (1:100, v/v) (Sigma), then washed with PBS and resuspended in 200 µl PBS for analysis by Flow Cytometry on a FACS Caliber Instrument (Becton Dickinson) using Cell Quest software. Monoclonal antibodies specific for CD34, CD68, SDF-1, CD45 and CD20 were purchased from Becton-Dickinson BioSciences (San Jose, USA).

2.6. Secreted levels of angiogenic cytokines

We examined the secretion of pro- and anti-angiogenic molecules including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and thrombospondin-1 (TSP-1). In the conditioned medium (CM) supernatants collected from both CLL B-cells alone, MSE alone, or MSE and CLL B-cells in co-culture, VEGF and bFGF were measured using Quantikine kits (R&D systems, Minneapolis, MN) and TSP-1 using Cytimmune (Maryland, MA) according to the manufacturer's instructions after 24 and 72 h culture.

3. Results

3.1. Characteristics of CLL bone biopsy derived marrow stromal elements

All the bone biopsy derived stromal cultures were inspected weekly under phase contrast microscopy. The average time for biopsy fragments to firmly attach to the culture plate was 2–3 weeks. Once attached, cellular elements growing in a “web-like” pattern appeared to be generated from the biopsy fragments (Fig. 1). By week four, a multiplicity of cell morphologies were present (Fig. 1). Morphologically, the cell types routinely present were fibroblasts, macrophages, dendritic cells, lymphocytes, and adipocytes (data not shown). Immunohistochemistry was used to further characterize these cells and revealed a very heterogeneous cellular population. Thus, consistent with the findings of Raza, et al. [22], cells reactive to antibodies specific for CD34, CD68, SDF-1, CD45 and CD20 were repetitively detected in both normal and CLL derived bone biopsy cultures (data not shown). Hereafter in this text, we refer to these cells as CLL derived stromal cell cultures or human MSE.

In some experiments, plates were used between 8 and 12 weeks (at confluence) to study CLL B-cell–stromal cell interactions (described below) while other samples were maintained in culture. The long-term nature of bone biopsy derived stromal cell cultures were assessed by

determining both how long primary marrow cultures could be maintained and whether secondary cell culture could be established. Primary CLL derived stromal cell cultures could be routinely cultured for >6 months. At the time of this report some cultures have been maintained for >1 year, although most have been maintained between 6 and 24 weeks. In other experiments, we were able to freeze the stromal cell cultures and use them again as functioning CLL derived stromal cell cultures after thawing. Additionally, we have been able to place the bone biopsy fragments into liquid nitrogen immediately after collection from patients and thaw them when needed to seed new bone marrow stromal cell cultures (data not shown).

3.2. CLL derived MSE protect CLL B-cells from spontaneous and drug-induced apoptosis

Having established these stromal cell cultures, we next evaluated if they could rescue CLL B-cells from spontaneous apoptosis. CLL B-cells at $5 \times 10^6/\text{ml}$ were co-cultured in direct contact with confluent CLL derived stromal cells or in medium alone. CLL B-cell viability was assessed at 24 and 72 h using annexin/PI staining. Spontaneous apoptosis was significantly reduced for the BM exposed CLL B-cells compared to those cultured in medium alone (Fig. 2). We next tested whether CLL derived stromal cell cultures could protect CLL B-cells from drug induced apoptosis. CLL B-cells were cultured with chlorambucil in combination with either medium alone or in combination with CLL derived stromal cell elements for 24 and 72 h. Exposure to MSE decreased the *in vitro* sensitivity of CLL B-cells to chlorambucil. Thus, after 24-h culture, apoptosis levels were reduced from a mean of $58.8\% \pm 7.8$ for CLL B cells cultured with $1 \mu\text{M}$ chlorambucil in medium alone to $39.8\% \pm 3.8$ for CLL B-cells cultured with $1 \mu\text{M}$ chlorambucil in co-culture with MSE, ($n = 5$; $p = 0.001$). In separate experiments with CLL B-cells exposed to MSE derived from normal donors or to the stromal cell line HS-5; we also noted significant protection from apoptosis (data not shown).

Due to prior work demonstrating the vulnerability of CLL B-cells to oxidation [25,28–30], we next evaluated if MSE could rescue CLL B-cells from pharmacologic agents that induce cell death by generating oxidative stress. Glucose oxidase was used as an oxidative challenge in these experiments due to its ability to generate a pure source of peroxides and induce cell death in CLL B-cells [27,28]. CLL B-cells were exposed to glucose oxidase (1 unit) for 30 min, washed with PBS and then cultured with or without MSE or HS-5 stromal cells. Both MSE from CLL patients and the HS-5 stromal cell line provided a similar degree of protection against apoptosis under these conditions (Fig. 3). In these experiments, we also used transwells to allow exposure of the CLL B-cells to factors secreted by MSE but preventing physical contact with MSE. These experiments demonstrated that soluble factors from both CLL derived MSE and HS-5 stromal cells protected CLL B-cells from apoptosis, although to a lesser degree than under conditions that permitted direct physical contact between CLL B-cells and stromal cells (Fig. 3 and data not shown). In similar experiments, we have found that HS-5 stromal cells release a soluble factor which protects CLL B-cells from adaphostin induced cell death, another drug that we have shown works via induction of ROS [25]. In support of the transwell experiments, we found a similar degree of protection against agents that induce oxidative stress when CLL B-cells were cultured in media conditioned by HS-5 cells for 24 h (data not shown). These findings demonstrate that secreted factors from MSE and HS-5 cells can generate a substantial degree of protection against CLL B-cell death induced by oxidative stress.

Given the ability of MSE to provide marked protection for spontaneous and drug-induced apoptosis, we next evaluated the potential mechanism by which this protection occurred by evaluating if culture of CLL B-cells with CLL derived MSE increased levels of anti-apoptotic proteins in CLL B-cells. After 24-h culture of CLL B-cells with CLL derived MSE, we found marked increases in the levels of multiple anti-apoptotic proteins including Mcl-1, XIAP, Bcl-2, and survivin (Fig. 4).

3.3. Angiogenic potential of CLL derived stromal cells

We previously reported that CLL B-cells possess a VEGF based angiogenic pathway that supports their survival [26,31]. In addition, we have shown CLL B-cells actively secrete both pro- and anti-angiogenic cytokines [32], and we and others have demonstrated these cytokines affect anti-apoptotic protein levels [32–35]. To evaluate the dynamics of vascular cytokine secretion when CLL B-cells are cultured with CLL derived stromal cells, we added CLL B-cells to culture medium alone or CLL derived stromal cells. After 24 and 72 h, the culture medium was harvested in order to measure secreted levels of pro- and anti-angiogenic cytokines. Fig. 5A–C show the results of these experiments. We found that, when cultured without CLL B-cells, CLL derived stromal cells secreted VEGF, TSP-1 and small but detectable levels of bFGF. In contrast, when cultured without CLL derived stromal cells, CLL B-cells secreted modest amounts of bFGF and TSP-1 with small but detectable amounts of VEGF. When CLL B-cells were cultured in combination with the CLL derived MSE, dramatic more than additive changes in levels of secreted angiogenic cytokines were observed. The level of secreted VEGF decreased (reduction of 70–72%, Fig. 5A) while there was a dramatic increase in the secreted levels of bFGF (25 to 30 fold increases, Fig. 5C). In co-culture as compared to MSE alone. Of interest, TSP-1 levels were stable at 24 h and significantly decreased at 72 h (Fig. 5B). On balance, we interpret this combination of events on co-culture to indicate that when CLL B-cells are cultured with bone biopsy derived MSE the interaction drives the regulation of angiogenic cytokines in favor of angiogenesis (i.e., increased bFGF along with decreased TSP-1).

4. Discussion

In this study we have utilized a novel bone biopsy technique to develop bone marrow stromal cell cultures from CLL patients that permit functional *in vitro* assays of leukemic cell–stromal interactions. This has provided us with an opportunity to study the ability of these cell cultures to modulate spontaneous apoptosis and rescue CLL B-cells from drugs that exert their cytotoxic effects through a variety of mechanisms. The stromal cells cultures were found to be robust, long-lived, and able to sustain apoptosis resistance in primary CLL B-cells. In addition, we found that protection from drug-induced cell death by CLL derived bone marrow stromal cells or MSE was observed for both alkylating agents and drugs that bring about cell death by inducing oxidative stress.

Importantly, these experiments suggest that stromal protection is mediated, in part, by soluble factors produced by marrow stromal elements. The nature of the soluble factor(s) mediating this protection is currently not known, but preliminary work by us indicates that the molecular weight is >10 kDa. The candidates for this soluble factor are multiple and include shed receptors (CD25, CD23) or certain cytokines including IL-4, IL-2, BLyS, APRIL, bFGF and VEGF. The latter cytokines have all been shown to enhance CLL B-cell survival or induce drug resistance. Further work is ongoing to identify the factor(s) since maneuvers to reduce the activity or levels of the protective factor may augment the efficacy of some therapeutic approaches. Similar to other investigators [36,37], we have not seen the same kind of protection with MSE soluble factors for CLL B-cells exposed to fludarabine where physical contact between stroma and CLL B-cells appears to be necessary for protection to occur (data not shown). Therefore, the mechanisms by which MSE protect CLL B-cells from drug induced death appears to depend to a certain extent on the drugs mechanism of action.

We have also demonstrated that interactions between CLL B-cells and primary marrow stromal elements derived from CLL bone biopsy specimens affect levels of anti-apoptotic proteins and engage in functional interactions that induce dramatic alterations in the levels of secreted pro- and anti-angiogenic cytokines. We examined the angiogenic cytokine profile for MSE and CLL B-cells when cultured together for several reasons. First, there is extensive

neovascularization in both marrow and nodes of CLL patients. Second, serum/plasma levels of angiogenic cytokines have been shown to correlate with clinical outcomes in patients with CLL [16,38,39]. Third, signaling through angiogenic receptors appears to relate to a number of biologically important events in the survival of CLL B-cells [17]. Specifically, we have defined the presence of a VEGF-based autocrine pathway in CLL B-cells [17,26,31]. Neovascularization and angiogenic signaling depends on the balance of pro- to anti-angiogenic cytokines, the so-called angiogenic switch. Thus, our finding that interactions between CLL B-cells and MSE lead to an increase in some pro-angiogenic cytokines (bFGF) along with a diminution of at least one anti-angiogenic cytokine (TSP-1) is notable and identifies one mechanism by which stroma may enhance CLL B-cell survival. Since previous work has also shown that bFGF can increase CLL B-cell resistance to fludarabine [35], these findings also have implications for drug resistance. Accordingly, therapeutic strategies aimed at neutralizing angiogenic cytokines may enhance the benefits of purine nucleoside therapy [17].

Finally, we believe, the use of bone biopsy derived stromal cells is an advance over the use of bone marrow aspirate cells or stromal cell lines for studying interactions between CLL B-cells and marrow stromal cells. This approach recapitulates the cellular heterogeneity of the marrow stromal and provides a long-lived culture system that can be regenerated by secondary culture or even after long-term tissue storage. Because of the ability to sustain these stromal elements, this type of stromal cell culture system allows the study of CLL B-cells isolated from the same patient over time with autologous bone marrow without requiring repeat marrow biopsies. This latter approach will help determine if marrow stromal elements have a greater or reduced ability to alter CLL B-cell survival as the clonal B-cells evolve in a given patient. While a direct comparison of normal versus CLL derived stromal cells (i.e., MSE) was beyond the scope of this study, we did not find any dramatic differences in the ability of these two sources of stroma to promote CLL B-cell survival (data not shown). However, in future studies, we intend to do additional studies to more intensively compare and contrast the features of the normal vs. leukemic derived stromal cells for their ability to modulate CLL B cell apoptosis.

In total, these studies demonstrate the utility of a bone biopsy derived stromal cell system to study the intimate cross-talk that occurs between CLL B-cells and the marrow microenvironment. We find that this interaction influences CLL B-cell resistance to both spontaneous and drug induced apoptosis. While the mechanisms for these effects are shown to be complex, it is apparent that this leukemic cell–stromal cell model will yield more insights that reflect *in vivo* mechanisms related to CLL disease progression. In turn these insights may ultimately provide a valuable framework for rational approaches to test and evaluate drugs that interrupt leukemic cell–stromal interactions to achieve more effective removal of CLL B-cells.

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Fig. 1.

Features of the bone biopsy derived stromal cells (MSE) from CLL patients. This figure shows sequential growth radiating out from a bone spicule harvested from a CLL patient over a 4-week period. Note the gradual increasing confluence of a heterogeneous cell population over time. Magnification was 10 $\times$. In the 4-week culture of stromal cells note the dense dark components which are small bone spicules. Note also the very tight organization of cells within this one radiating group of cells with less confluent cells at either side of the radiating cell group.

Fig. 2.

MSE protects CLL B cells from spontaneous apoptosis. The medium columns represent CLL B cell levels of apoptosis when cells were cultured in medium alone for 24 or 72 h as described in methods. MSE columns represent CLL B cell apoptosis when CLL B-cells were cultured with MSE for 24 or 72 h as described in methods. Data is presented as mean±standard error of mean (S.E.M.) for five separate experiments. *P* values represent paired *t* test for the apoptosis data in medium vs. MSE cultures.

Fig. 3.

Viability of CLL B-cells after exposure to glucose oxidase followed by culture with or without human MSE. CLL B-cells ($n = 6$) were cultured at 37 °C for 30 min in serum free medium (control) or medium containing 1 unit of glucose oxidase (GO). After 30 min, cells were washed with PBS. Control cells were placed in wells containing serum free medium (control). Cells exposed to GO were placed in wells with one of four environments: serum free medium alone (GO-media alone), in physical contact with HS5 stromal cells (GO-HS5), in physical contact with primary human bone marrow (GO-CLL stromal cell-contact), and in transwell with primary human bone marrow which permitted exposure to marrow conditioned medium but did not allow physical contact between CLL B-cells and marrow stromal cells (GO-CLL stromal cell-transwell). After culture for 4 or 8 h at 37 °C, cells were harvested and washed with PBS. The viability of CLL B-cells was assessed by flow cytometry using annexin/PI. Y-bars represent standard error bars.

Fig. 4.

CLL B cells have induction of anti-apoptotic proteins after culture with human MSE. In this experiment CLL B-cells from two CLL patients were cultured in medium alone (-) or in physical contact with CLL MSE (+) for 24 h. Cell lysates were prepared and subjected to immunoblot as per methods. Specific monoclonal antibodies were used to assess the levels for XIAP Mcl-1, Bcl-2 and survivin. Note the increase in all anti-apoptotic proteins when CLL B cells are cultured with MSE. Also shown are the levels of actin for each cell lysate indicating equal loading for each lane.

Fig. 5. The MSE–CLL B-cell interaction induces a pro angiogenic phenotype or “switch” CLL B cells ($n = 4$) were added to confluent MSE for 24 or 72 h at which time the CM was harvested for ELISA determination of VEGF (A), TSP-1 (B) and bFGF (C) levels. MSE represents marrow stromal elements cultured alone; Nil represents CLL B cells cultured in medium alone; and CLL + MSE represents MSE and CLL B-cells cultured together. Note the dramatic decrease in TSP-1 at 72 h for CLL + MSE co-cultures (B) compared to the obvious increases in bFGF for CLL plus MSE co-cultures at 24 and 72 h (C).