Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 391-398

OSTEOBLASTS RELEASE A SOLUBLE FACTOR THAT MODULATES IMMUNOGLOBULIN SECRETION BY MATURE B CELLS

Dafna Benayahu[1*], Irit Altboum[2], Dov Zipori[1], Shlomo Wientroub[3,4] and Israel Zan-Bar[2]

[1]Department of Cell Biology, Weizmann Institute of Science, Rehovot , Israel

[2]Department of Human Microbiology , [3]Division of Orthopedics , Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

[4]Department of Pediatric Orthopedics, Dana Children's Hospital, Tel-Aviv Medical Center, Tel-Aviv , Israel

Received June 2, 1993

SUMMARY: An osteogenic member, MBA-15 cell line, from marrow stroma compartment, was examined for its ability to regulate mature lymphoma B cell line functions. The effect of MBA-15 cell secreting factor/s on cell proliferation, immunoglobulin secretion and isotype switch of 29M4.1 IgM^+ mature B cell line was examined. The factor causes temporal arrest in cell proliferation, augments immunoglobulin secretion and has no effect on isotype switching. The results revealed a direct effect of the MBA-15 factors on the B cell functions. Factors secreted by other osteoblastic cells (ROS 17/2.8 and MC-3T3-E1) possess the same biological activities. In contrast, factors secreted by an endothelial adipocyte stromal cell line 14F1.1 lack these properties.

All blood cells are formed within the bone marrow with the exception of T lympocytes. Multipotential stem cells, from which all blood cells are derived, are preferentially located in the subendosteal area of bone marrow adjacent to the bone surface (1). The cell adhesion molecules which attract and maintain these cells in this location are only partially understood (2). The stromal cells are multifunctional with respect to the hemopoietic lineage cells. The stroma cells support the hemopoietic cells' proliferation and maturation and are known to constitutively produce many cytokines. The hemopoietic cells display receptors for these cytokines, which indicates that they are responsive to these local stimuli.

* To whom reprint requests should be addressed.

0006-291X/93 $4.00

Immature precursors of B lymphocytes have been localized nearby the bone tissue and it is believed that they move progressively towards the center of the bone as they divide and differentiate (3). In that site highly specialized B lymphocytes are produced together with cells of several other lineages via a process which must have unique features.

Study of the molecular basis of B cell regulation revealed cytokines which are selectively used in the bone marrow and in the periphery. These events probably depend on the physical and molecular interactions between B cell progenitors and components of the microenvironment, i.e. stromal cells through cell-cell interactions and/or matrix bound soluble factors secreted by these cells. Many factors secreted by stromal and other cells are known to be involved in regulation of progenitor B cells as well as of mature B cells [interleukins (IL) IL-4, IL-6, IL-11, TGFß etc.] (4). The task of defining the marrow member/s in the microenvironment involved in B lymphocyte regulation is a complex one and may be multifactorial.

In this study we examine the ability of stromal cells to regulate mature B cell functions. The modulation of B lymphocyte differentiation is represented in immunoglobulin (Ig) secretion and isotype switch under appropiate stimulatory signal. We examined the effects of secreted factor/s from marrow stromal-derived osteogenic cells (MBA-15) (5) on induction of differentiation in 29M4.1 B lymphoma cell line (6). This IgM^+ B lymphoma cell line differentiates upon stimulation to IgA bearing cells and in IgM and IgA secretion (6,7).

MATERIAL and METHODS

Cell lines and culture conditions. 29M4.1, an IgM^+, IgA^- B cell lymphoma line (6,7) was produced as previously described via sorting and cloning of I.29 lymphoma cells (8-10). 29M4.1 cells were maintained in RPMI-1640 (Gibco, USA) supplemented with 10% fetal calf serum (FCS, Bio-lab, Israel), Hepes buffer (10 mM), 2-mercaptoethanol (0.05 mM) and L-glutamine (2 mM). The stock cells were grown in tissue culture dishes (Nunc, Denmark) at 37°C with 7.5% CO_2 in air.

Stromal cell lines, derived from mouse bone marrow used in this study were the MBA-15 osteoblastic cell line (5) and the 14F1.1 cloned endothelial-adipocyte cell line (11). The 14F1.1 cell line has a major role in regulation of hemopoiesis, as was previously shown in long-term cultures. Other non stromal osteoblastic cell lines were mouse calvaria derived MC3T3-E1 and rat osteosarcoma cells (ROS 17/2.8). All cell lines were seeded in tissue culture dishes in DMEM (Bet-Haemek, Israel) containing high glucose supplemented with 10% FCS. Cultures were incubated at 37°C in 10% CO_2 in air.

Conditioned medium (CM). All cell lines were seeded at $1.5x10^5$ cells/ml into 60-mm culture dishes in DMEM with 10% FCS and were grown to confluence. The medium was then removed and fresh medium was added to the culture dishes for 24 hours. These conditioned media (CM) were collected, centrifuged, millipore filtered (0.45 μm) and stored in small aliquots at -20°C.

Experimental protocol. $5x10^4$ 29M4.1 cells were cultured in 96-well microtiter plates with various concentrations of CM with or without 10 μg/ml of lipopolysaccharide (LPS, Escherichia coli 055:B5, Difco Laboratories, Detroit MI) for 4 days. Cell culture supernatants were harvested and IgM and IgA

concentrations were measured by ELISA. Parallel cultures were pulsed with [^{3}H]-thymidine for the last 4 hours and then harvested for proliferation determination.

Measurement of isotype concentrations in the culture supernatants. Isotype concentrations were measured by a solid-phase immunoassay (12). Nunc Immunoplate Maxisorp 96-well, flat bottomed ELISA plates (Nunc, Denmark) were coated with goat anti-mouse Lambda (Nordic Immunological Laboratories, Netherlands). Biotin conjugated goat anti-IgM or goat anti-IgA (Amersham, England) and streptavidin conjugated with alkaline phosphatase (Amersham, England) were then used for measurement of IgM and IgA respectively. The dye developed during the reaction was read using a microplate reader (Microplate Autoreader Bio-Tek Instrument, USA). Ig concentrations were determined by extrapolation from standard curves by using purified myeloma proteins of known concentration in every assay. The final results were calculated as ng/ml divided per cpm of [^{3}H]-thymidine incorporation since the CM effects the rate of cell proliferation. Calculation of the data as ng/ml per cell number (via counting viable cells at each experiment), demonstrated the same results.

Cell cycle analysis. $5x10^6$ 29M4.1 cells were cultured in 24-well plates in a volume of 1 ml with various concentrations of stromal cell CM with or without 10 μg/ml of LPS, for 48 hours. Percentage of the cells in the G_0/G_1, S, G_2/M phases was determined by analysing the cell nuclei stained with Propidium Iodid (PI) on a flow cytometer apparatus, FACS 440 Becton Dickenson, San Jose CA, USA (13).

Statistical analysis. The data were expressed as the mean ± SEM. The significance levels analysis of variance (ANOVA).

RESULTS

In view of the multiplicity of factors able to affect B cell function in vivo, we examined the stromal cell-dependent B cell proliferation and differentiation responses. We first examined the effect of CM harvested from marrow stromal derived osteogenic cells (MBA-15) on the proliferation of 29M4.1 cells. The results revealed that MBA-15 CM has a direct inhibitory effect on the 29M4.1 cell growth in a dose-dependent pattern (Fig.1A). Cell cycle analysis was in correlation to the proliferation rate, an elevation in the G_0/G_1 phase and a decrease in the G_2/M phase of 29M4.1 cells in response to MBA-15 CM (Table 1). In parallel, we have monitored the effect of MBA-15 CM on Ig secretion and isotype switch of 29M4.1 cells. The results demonstrated the existence of a remarkable direct stimulatory effect on IgM and IgA secretion of 29M4.1 cells in a dose-dependent manner (1% up to 20%) (Fig 1B,C). Addition of MBA-15 CM was found to be synergistic to the effect of LPS in IgM and IgA secretion. 5-20% CM couse a 10-20 fold elevation in Ig secretion of LPS stimulated cells. The maximal effects were detected at a concentration of 5% CM. The same pattern of elevation was demonstrated both in IgM and IgA secretion and no changes in the relative proportion of IgM/IgA secretion were noticed.

No effect on Ig secretion of 29M4.1 cells was revealed with CM obtained from another marrow stromal cell type, endothelial-adipocyte, 14F1.1 (Fig.2 A,B).

Since the CM harvested from osteogenic cell MBA-15 had shown the ability to modulate the 29M4.1 B lymphocyte proliferation and Ig secretion, we examined CM

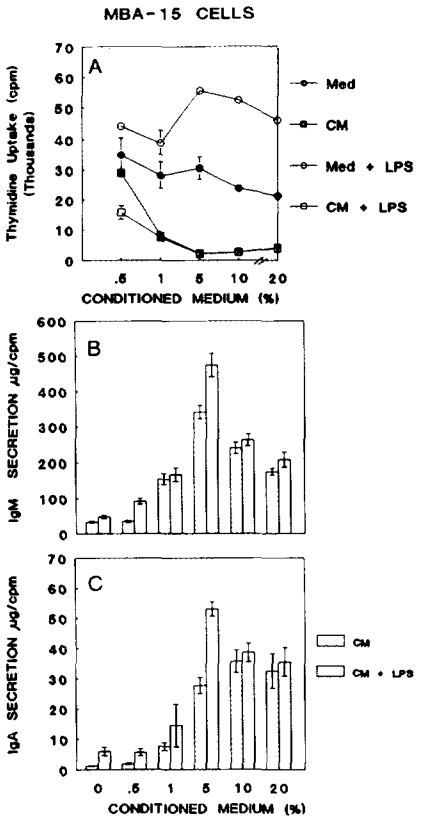

Fig.1. The effects of MBA-15 cell CM on proliferation and Ig secretion by 29M4.1 cells. A. - Rate of proliferation as measured by [^{3}H]-thymidine uptake. B, C. - IgM and IgA concentration in cell culture supernatants measured by ELISA. The results shown are mean ± SEM of ng/ml per cpm as described in the "Material and Methods". The experiments were performend in 3-6 wells per group and the results are of one representative experiment. The CM added from 1% to 20% effects are significantly different ($p<0.05$) compared to the control level.

Table I: The effect of MBA-15 CM on the cell cycle phases of 29M4.1 cells

	G_0/G_1	△	S	G_2/M	△
Medium	36.7		44.9	18.4	
CM	43.1	17.0	43.4	13.5	-36
Medium + LPS	33.6		45.9	20.5	
CM + LPS	36.3	7.7	47.9	16.2	-26

$5x10^6$ cells were cultured with CM with or without 10 µg/ml of LPS for 48 hours. Percentages of the cells in GO/G1, S, G2/M phase were determined by analysis of PI stained nuclei on a flow cytometer apparatus. △ - The difference in treated cells compared to control levels.

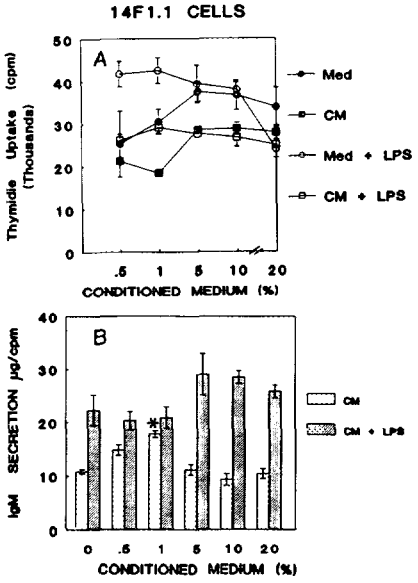

Fig.2. The effects of 14F1.1 cells CM on proliferation and IgM secretion by 29M4.1 cells. A. - Rate of proliferation as measured by [^{3}H]-thymidine uptake. B.- IgM concentration in cell culture supernatants measured by ELISA. As in Fig. 1. The CM effect is significantly different ($p<0.05$) compared to the control level marked "*".

harvested from other osteoblastic cell lines, MC3T3-E1 mouse calvaria and ROS 17/2.8 rat osteosarcoma. Addition of CM of MC3T3-E1 or ROS 17/2.8 cells to 29M4.1 cells decreased the rate of cell proliferation, induced IgM and IgA secretion and synergized the effect of LPS in a dose-dependent manner with maximum effect at 5% (Fig. 3). All CM harvested from other osteoblastic sources demonstrated effects similar to those of MBA-15 CM on 29M4.1 cells, i.e., induction of temporal cell proliferation arrest (Fig. 3A,D) and augmentation of antibody secretion (Fig. 3 B,C,E,F) in a dose-dependent manner. These results indicate that osteoblastic cells secrete soluble factors that can directly induce IgM and IgA secretion by 29M4.1 B cells. The stimulatory effects were increased by 10-20 fold in a dose-dependent pattern. In addition CM obtained from these cell lines synergized the effect of LPS.

In order to rule out the existence of non-specific inflammatory agents in the CM of various cell lines we examined these media for mycoplasma and endotoxin activity. All CM batches examined were found to be mycoplasma-negative and endotoxin-free.

DISCUSSION

The stromal cells are multifunctional with respect to various stages of hemopoietic lineage differentiation. The adherent stroma cells are capable of

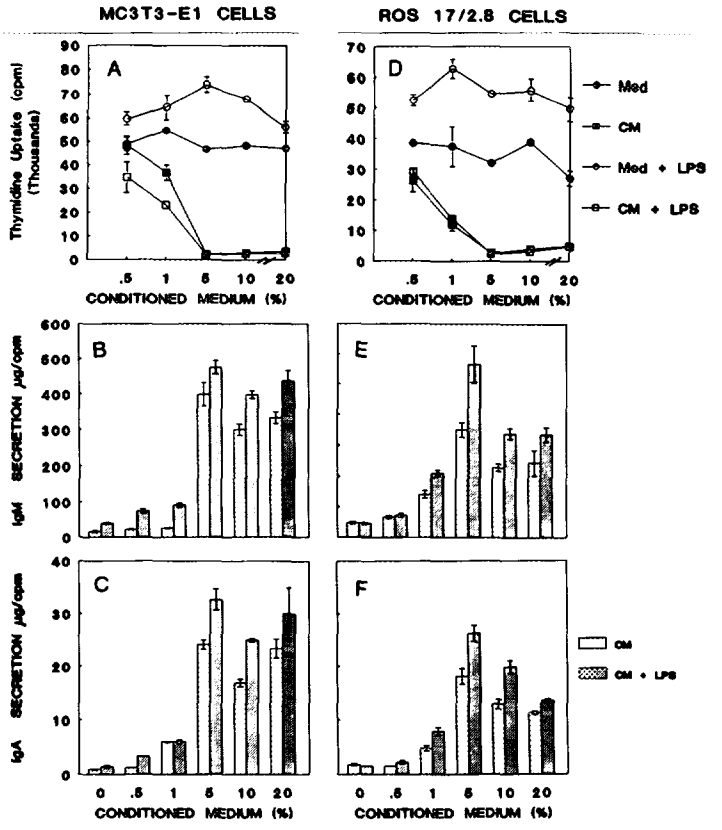

Fig. 3. The effects of MC3T3-E1 and Ros 17/2.8 cells CM on proliferation and Ig's secretion by 29M4.1. A.,D., - Rate of proliferation as measured by [^{3}H]-thymidine uptake. B.,E.- IgM and C.,F.- IgA concentration in cell culture supernatants measured by ELISA. As in Fig. 1.

producing various cytokines that may be partially accounted for these supportive effects (14,15). The function of individual stromal cell types is probably associated with local microenvironmental stimuli as well as with systemic inflammatory regulators. This multiplicity of inducers raised the question of how these cytokines influence the complex orchestration between proliferation and differentiation of various cell types (15,16).

In this study we examined the activities of factor/s secreted by well defined stromal cell type on mature B lymphocyte functions. We demonstrated that substances produced by marrow stroma osteogenic cells, MBA-15, as well as by other types of osteoblasts cause arrest of cell proliferation and induce Ig secretion with no effect on isotype switch. On the contrary, substance/s produced by the endothelial-adipocyte stromal cell, 14F1.1, did not express similar activities.

The 29M cell lines that originated fron I.29 cells, express surface Ig, B-220, Ly-1 and lack FcRγ and murine plasma cell markers (6,7). The development of these new sets of IgM, IgG1, IgG2a, IgG2b, IgE and IgA sublines enabled us

to study the effect of various known and unknown cytokines on cell proliferation, Ig secretion and isotype switch. We previously found that these cell lines can respond to LPS, recombinant interleukins (rIL), rIL-4, rIL-5, IFN-τ, or TGFß2, with Ig secretion and with further downstream isotype switch (6,7). The effects of interleukins were found to be totally dependent on additional activation signals delivered by high doses of LPS. None of the mentioned factors have direct effects on cell growth, Ig secretion or isotype switch. In addition, we found that rIL-6 augments Ig secretion (submitted for publication), while rIL-2, rIL-3 or GM-CSF do not play any role in these cell lines' functions (unpublished data).

In the present study we demonstrated that the marrow stromal osteogenic cell line, MBA-15, is involved in the regulation of mature B cell functions. We also showed the effects of other osteoblastic cell lines on modulation of isotype secretion and on the rate of proliferation of 29M4.1 cells. In contrast to known cytokines, the CM from the osteoblastic cell lines mediated Ig secretion by themselves in a dose-dependent fashion. In addition, these CM synergized with LPS. The activity of CM harvested from various osteoblastic cell lines indicates that factor/s secreted by these cell types may have an essential role in mature B cell functions.

We have shown earlier that MBA-15 cells can affect the in-vitro growth of normal and malignant hemopoietic cells. MBA-15 cells produce and release constitutively M-CSF, IL-6 and GM-CSF which are involved in myeloid cell maturation (17). The possible action of M-CSF was ruled out since this factor is also secreted by 14F1.1 cells (18) which did not affect the 29M4.1 B cells. By using high concentrations of MBA-15 CM we observed restraining activity on myeloid cell proliferation and differentiation. These inhibitory effects involved direct and indirect cell to cell contact in coculture systems (17). ROS 17/2.8 cells are known to secrete factors with GM-CSF-like activity (19). Calvarial cells and MC3T3-E1 cells secrete M-CSF constitutively which can be elevated in the presence of LPS or 1,25-$(OH)_2D_3$ (20). In the MC-3T3-E1 cell line, GM-CSF is secreted only after LPS stimulation (21). This may imply that in-vivo, cells with osteoblastic phenotype do not secrete CSFs continually but rather in response to specific signals. Stromal osteoblastic cells MBA-15 secrete cytokines constitutively while other osteoblast-like cells secrete the cytokine in response to local and systemic stimuli.

The biological effects monitored in this study are thus unique and may be due to new cytokines that affect, by themself or in combination with other factors, the proliferation and Ig secretion of mature B cells.

ACKNOWLEDGMENTS

This work was supported in part by grants from the U.S. - Israel Binational Science Foundation (89-00482/2) to (S.W.), the Israel Cancer Research Fund, the

Israel Cancer Association and the Moise and Frida Eskenasy Institute for Cancer Research to (I.Z.B.).

REFERENCES

1. Lord, B.I. (1990) Intern. J. of Cell Cloning 8,317-331.
2. Tavassoli, M., and Minguell, J.J. (1991) Proceedings of the Society for Experimental Biology and Medicine 196, 367-373.
3. Jacobsen, K., and Osmond, D.G. (1990) Eur. J. Immunol. 20, 2395-2404.
4. Taga, T. and Kishimoto, T. (1993) FASEB J. 7,3387-3396.
5. Benayahu, D., Kletter, Y., Zipori, D. and Wientroub, S. (1989) J. Cell. Physiol. 140: 1-7.
6. Altboum, I., and Zan-Bar, I. (1992) Cells in Development and Disease, (L. Herzenberg et al, ED.), Vol 651, pp. 152-156. Annals of the New York Academy of Sciences.
7. Porat, Y., Altbaum, I., and Zan-Bar I. (In press) Cellular Immunology.
8. Sato, H., Boyse, E.A., Aoki, T., Iritani, C., and Old, L.J. (1973) J. Exp. Med. 138, 593-606.
9. Stavnezer, J., Radcliffe, G., Lin, Y.C., Nietupski, J., Berggren, L., Sitia, R., and Severison, E. (1988) Proc. Natl. Acad. Sci. USA 85, 7704-7708.
10. Alberini, C., Biassoni, R., DeAmbrosis, S., Vismura, D., and Sitia, R. (1987) Eur. J. Immunol 17, 555-562.
11. Zipori, D., Friedman, A., Tamir, M., Silverberg, D., and Malik, Z. (1984) J. Cell. Physiol. 118, 143-152.
12. Snopper, C.M., and Paul, W.E., (1987). J Immunol. 139, 10-16.
13. Videlov, L.L., and Christensen, I.J. (1990) Cytometry 11, 753-770.
14. Kincade, P.W. (1992) In Growth Factors in Haemopoiesis (B.I. Lord, T.M. Dexter, Ed.) Vol. 5(3), pp. 575-598. Baillier's Clinical Haematology.
15. Zipori, D. (1990) Cancer Cells 2, 205-211.
16 Zipori, D. (1992) FASEB J. 6, 2691-2697.
17. Benayahu, D., Horowitz M., Zipori, D., and Wientroub, S. (1992) Calcif. Tissue Int. 51, 195-201.
18. Zipori, D., and Lee, F. (1988) Blood 71, 589-596.
19. Weir, E.C., Insogna, K.L., and Horowitz, M.C. (1989) Endocrinology 124, 899-904.
20. Elford, P.R., Felix, R., Cecchini, M., Trechsel, U., and Fleisch, H. (1987) Calcif. Tissue Int. 41, 151-156.
21. Horowitz, M.C., Coleman, D.L., Ryaby, J.T., and Einhorn, T. (1989) J Bone Mineral Res 4, 911-919.