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OKADAIC ACID IS A POTENT INDUCER OF AP-1, NF-κB, AND TUMOR NECROSIS FACTOR-α IN HUMAN B LYMPHOCYTES

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Treatment of human B lymphocytes with an optimal concentration of okadaic acid, an inhibitor of phosphatases 1 and 2A, resulted in the induction of the transcription factor, AP-1 and a marked increase in NF-κB levels. In contrast, no effect on the levels of the octamer binding proteins, Oct-1 or Oct-2, were found. Since both AP-1 and NF-κB have been reported to be important in the induction of the tumor necrosis factor-α (TNF-α) gene we examined the effects of okadaic acid on TNF-α mRNA levels. Treatment with okadaic acid resulted in a striking increase in TNF-α mRNA transcripts within 1 h of stimulation and large amounts of TNF-α were released into the culture media. Although okadaic acid provides a potent inductive signal for AP-1 and NF-κB it did not induce either B cell proliferation or immunoglobulin secretion.

The study of B cell activation has largely relied on three different classes of activation stimuli either the crosslinking of surface membrane Ig, the direct stimulation of protein kinase C via phorbol esters, or via several monoclonal antibodies which recognize cell surface determinants presumably important in B cell activation. Each of these methods have been shown to activate protein kinases which mediate at least a portion of the activation signal (1-4). In contrast, the role of cellular phosphatases in cell activation is less well understood. The recent observation that CD45 is a tyrosine specific phosphatase and that it is involved in the regulation of lymphocyte activation has focused more attention on their role (5,6). Evidence that CD45 is important in B cell activation includes the observation that antibodies to CD45 inhibit anti-μ induced c-myc induction and B cell proliferation (7). Besides the data on tyrosine phosphatases there are data implicating serine/threonine phosphatases in the regulation of cell activation and proliferation (8).

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One of the reagents used to evaluate the role of serine/threonine phosphatases in these processes has been okadaic acid (8,9). Okadaic acid is a complex polyether derivation of a 38-carbon fatty acid synthesized by marine dinoflagellates that accumulate in feeding organisms such as black sponge (10). It has been shown to be a potent tumor promoter; however, in contrast to phorbol ester tumor promoters it does not bind to protein kinase C, but rather is a potent and specific inhibitor of the serine/threonine phosphatases 1 and 2A (PP-1 & PP-2A) (11,12). When added to intact cells the inhibition of protein phosphatases results in the unopposed activity of constitutively present protein kinases which leads to enhanced phosphorylation of many protein kinase substrates. Several recent studies have shown that treatment of the T cell lines, Jurkat and EL-4, with okadaic acid results in increased AP-1 levels (13,14) and increased mRNA levels of members of the fos and jun families of proto-oncogenes whose protein products interact to form AP-1 (13). Furthermore, treatment of Jurkat cells with okadaic acid induces another transcription factor, NF-κB (15). Those results suggested an important role for PP-1 and PP-2A in T cell activation. This study was undertaken to examine the effects of inhibiting PP-1 and PP-2A on B lymphocyte activation and subsequent proliferation and Ig production.

MATERIALS AND METHODS

B Cell Preparation and Cultures. B cells were prepared from human tonsils as previously described (16). B cells (0.5 x 10^6/ml) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and the antibiotics; penicillin, streptomycin, and gentamicin. Okadaic acid was provided by H. Fujiki (National Cancer Research Institute, Tokyo, Japan) and used at 70 ng/ml. Cycloheximide (Sigma Chemical Co., St. Louis, MO) was used at 10 μg/ml. In some experiments B cells were stimulated with 0.01% formalin treated SAC (Bethesda Research Lab., Gaithersburg, MD) and 100 units of recombinant IL-2.

Nuclear Protein Extraction and Mobility Shift Assay. Nuclear proteins extracts from B cells were prepared as previously described (17). Protein concentrations were quantitated using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). The binding reactions for the mobility shift assays were performed with 10 μg of nuclear protein, 17 μl of binding buffer (Stratagene, La Jolla, CA) and 50-100 pg of double stranded oligonucleotide end-labelled with ^{32}P. In some reactions a 50 fold molar excess of an unlabelled probe was added to show specificity. Samples were run on 5% polyacrylamide gels at 120V for approximately 2 h. Autoradiographs were exposed for 4-16 h. The oligonucleotides were synthesized on a 381 A DNA synthesizer (Applied Biosystems, Foster City, CA) according to published sequences (18-20).

RNA Isolation and Northern Blot. Cytoplasmic RNA was size fractionated on a 1.0% formaldehyde-agarose gel, transferred to nitrocellulose, ultraviolet cross-linked, and hybridized with a ^{32}P labelled tumor necrossis factor-α (TNF-α) cDNA probe as previously described (21).

Assays for B Cell Proliferation, Ig Production, and TNF-α Secretion. DNA synthesis was measured by determining the amount of ^3H-thymidine incorporated (10^5 cells/200 μl) over the last 8 h of the 72 h culture period using standard liquid scintillation counting techniques. Ig production was measured 6 days after initiation of the culture as previously described (21). TNF-α secretion into the supernatant was determined using a commercially available ELISA kit (R&D Diagnostics, Minneapolis, MN) following the manufacturer's instructions.

RESULTS AND DISCUSSION

An AP-1 mobility shift assay was used to assess the levels of AP-1 in nuclear extracts from B cells stimulated with okadaic acid. Previous studies with a T cell line, Jurkat, and preliminary experiments with B cells have demonstrated that the optimal concentration of okadaic acid to induce AP-1 is approximately 70 ng/ml (13). Stimulation of human B cells with this concentration of okadaic acid resulted in the induction of AP-1 within 2 h with a continued increased at 4 and 6 h (Fig. 1A). Detection of the AP-1 protein was inhibited by the protein synthesis inhibitor, cycloheximide. This suggests that either the induction of AP-1 by okadaic acid is dependent on c-Jun and/or c-Fos protein synthesis or, alternatively, protein synthesis is required for formation or transport of AP-1 into the nucleus. Stimulation of purified human B lymphocytes with 70 ng/ml of okadaic acid resulted in the induction of c-fos and c-jun mRNAs. Both c-fos and c-jun mRNAs were induced within 1 to 2 h and peaked at 4-6 h. Both mRNAs were induced even in the presence of cycloheximide (data not shown). These data are similar to studies with Jurkat cells treated with okadaic acid where induction of AP-1 was found to occur via an increased synthesis of c-Jun and c-Fos protein (13). This is in contrast to phorbol esters which induce AP-1 via both transcriptional and post-transcriptional mechanisms (22). Thus, inhibition of PP-1 and PP-2A in primary B cells results in a spontaneous induction of AP-1. This suggests that there are sufficient levels of protein kinases present in normal B cells that their unopposed activities result in the transcriptional activation of the c-fos and c-jun genes.

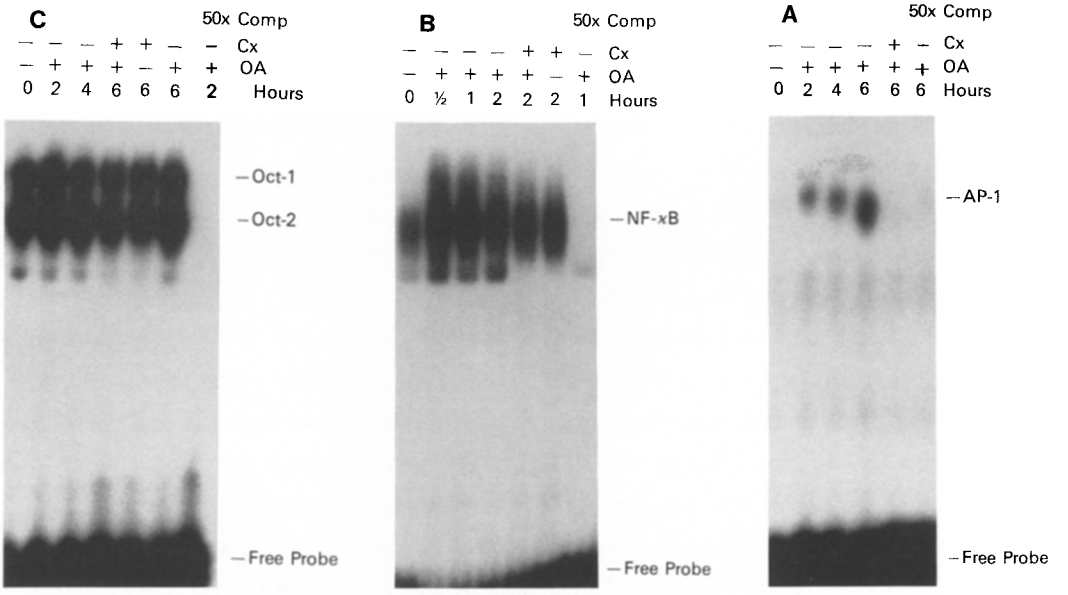

Figure 1. Effect of okadaic acid on AP-1, NF-κB, Oct-1, and Oct-2. Purified B cells were treated or were not with 70 ng/ml of okadaic acid for varying periods of time. Cycloheximide was added at 10 μg/ml to the cells 20 min prior to the okadaic acid where indicated. Nuclear proteins were extracted and incubated with ^{32}P labelled double stranded oligonucleotides containing the appropriate DNA binding site; AP-1 in panel A, NF-κB in panel B, and Oct-1 plus Oct-2 in panel C. Specific DNA-protein complexes were visualized by autoradiography and are appropriately labelled. The specificity of the binding was demonstrated by addition of 50 fold excess of unlabelled probe in the binding reaction (last lane in each case).

Another transcription factor NF-κB has been shown to be important in the induction of early response genes in lymphocytes (19). NF-κB is constitutively expressed in nuclear protein extracts from normal B lymphocytes; however, it can be further increased following B cell activation (23). Treatment of B cells with okadaic acid resulted in a striking and rapid increase in κB binding proteins. Within 0.5 h there was an approximately 4 fold increase. No further change in κB binding proteins was noted at 1 and 2 h post stimulation (Fig. 1B). The presence of cycloheximide did not alter the level of NF-κB noted after stimulation with okadaic acid although cycloheximide itself was a potent inducer. These rapid increases in κB binding activity following treatment with okadaic acid are unlikely to be accounted for by new transcription and translation of κB binding proteins; but rather likely results from post-translational modification of κB binding proteins present in the cytoplasm, their dissociation from the inhibitor IκB, and subsequent translocation to the nucleus. Consistent with this latter alternative was the observation that okadaic acid did not alter levels of p105 mRNA (24) which encodes p50 (data not shown)

In contrast to AP-1 and NF-κB, okadaic acid had no effect on the levels of the octamer binding proteins present in nuclear extracts from B cells which were or were not treated with okadaic acid. The same nuclear extracts previously examined for AP-1 and NF-κB were examined for the presence of Oct-1 and Oct-2 using an octamer probe (Fig. 1C). Both Oct-1 and Oct-2 were present constitutively in the nuclear extracts prepared from normal tonsil B cells (23). Treatment with okadaic acid in the presence or absence of cycloheximide did not significantly alter either Oct-1 or Oct-2 levels in B cell nuclear extracts. These results demonstrate that PP-1 and PP-2A are unlikely involved in the control of octamer binding in normal B lymphocytes. This does not rule out the possibility that PP-1 or PP-2A are important in the transcriptional activity of Oct-1 and Oct-2. In fact, differential phosphorylation of Oct-1 during the cell cycle by cdc2/H1 has been reported (25). cdc2/H1 is a kinase known to be activated by okadaic acid (26).

The induction of NF-κB and AP-1 by okadaic acid in normal B cells was accompanied by a marked increase in the levels of TNF-α mRNA. The TNF-α gene has been reported to have functionally significant NF-κB and AP-1 sites within its promoter region (27,28). Activation of B cells with SAC/IL-2 has been shown to induce TNF-α mRNA in normal B cells (21). A Northern blot with RNA prepared from highly purified tonsil B cells stimulated for varying periods of time with okadaic acid was probed with a TNF-α cDNA. There was a remarkable increase in TNF-α mRNA within 1 hour of stimulation which peaked at 4-6 h post-stimulation (Fig. 2A). The levels of TNF-α mRNA transcripts induced by okadaic acid compared to SAC/IL-2 were 8 fold higher as assessed by densitometric analysis (data not shown). The presence of cycloheximide did not inhibit the induction of TNF-α mRNA by okadaic acid. Both the rapid induction of TNF-α mRNA and its insensitivity to cycloheximide implicated NF-κB rather than AP-1 as the most important transcription factor in inducing expression of the TNF-α gene in B cells. Whether other transcription factors might also be important remains unknown; however, it is clear by inhibiting PP-1 and/or PP-2A that there is a potent induction of TNF-α mRNA levels in otherwise unstimulated B cells. In addition to the induction of TNF-α mRNA okadaic acid also promoted

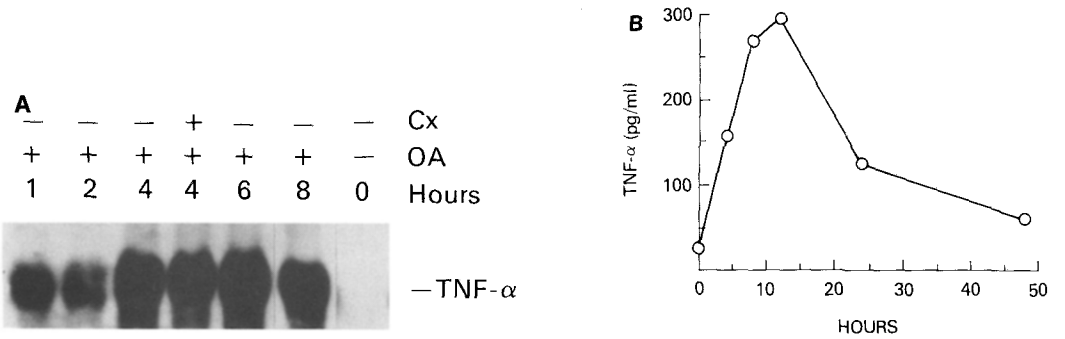

Figure 2. TNF-α mRNA induction and TNF-α protein secretion following stimulation of normal B cells with okadaic acid. A. Kinetics of TNF-α mRNA production following stimulation with okadaic acid. Northern blot of B cell RNAs prepared following stimulation with okadaic acid for varying times in the presence or absence of cycloheximide. B. Secretion of TNF-α from okadaic acid treated B cells. TNF-α levels in culture supernatants were quantitated using an ELISA based detection kit.

the release of TNF-α from B lymphocytes (Fig. 2B). TNF-α secretion peaked at 12 h and gradually declined.

In addition to inducing B cell activation, many of the B cell stimulatory signals also lead to B cell proliferation and, in the presence of various cytokines, the secretion of Ig into the culture media. The effect of okadaic acid on the incorporation of tritiated thymidine and Ig secretion were assessed in the presence or absence of the B cell stimulatory signals SAC and IL-2. No concentration of okadaic acid was found to induce either B cell proliferation or Ig secretion. The addition of okadaic acid (10 ng/ml to 50 ng/ml) to B cells stimulated with SAC/IL-2 markedly inhibited both B cell proliferation and B cell Ig secretion; lower concentrations had no significant

Table 1. Effects of Okadaic Acid on B Cell Proliferation and Differentiation[1]

Treatment of B cells	^{3}H thymidine uptake[2] x 10^{-3} cpm/well	IgG production[3] ng/ml
Medium	0.8 ± 0.2	96 ± 34
SAC/IL-2	18 ± 3	4200 ± 879
Okadaic acid		
(50 ng/ml)	1.2 ± 0.6	80 ± 29
(25 ng/ml)	0.9 ± 0.6	89 ± 42
(10 ng/ml)	1.3 ± 0.4	70 ± 28
(1 ng/ml)	1.1 ± 0.8	76 ± 28
(0.1 ng/ml)	1.1 ± 0.7	96 ± 36
SAC/IL-2 + Okadaic acid		
(50 ng/ml)	2.1 ± 1.2	89 ± 22
(25 ng/ml)	2.5 ± 1.2	112 ± 45
(10 ng/ml)	4.5 ± 1.5	212 ± 89
(1 ng/ml)	21 ± 6.7	3800 ± 545
(0.1 ng/ml)	24 ± 4.8	4800 ± 717

[1]The results represent the mean ± 2 s.d. of 3 independent experiments.
[2]Cells were cultured for 3 days. For proliferation assays cells were pulsed with ^{3}H thymidine 8 h prior to harvest. In the co-stimulation assay okadaic acid was added 8 h prior to SAC/IL-2.
[3]Cells were cultured for 6 days. Supernatant IgG was measured by ELISA.

effect (Table 1). While higher concentrations of okadaic acid are toxic, 10 ng/ml had little effect on cell viability when compared to B cells cultured in the absence of okadaic acid. Previous studies with okadaic acid have shown a complex effect on the cell cycle with both positive and negative effects noted (10,11,29). Since both phosphorylation and dephosphorylation events are crucial in the progression through the cell cycle, gross inhibition of PP-1 and PP-2A is likely to disrupt cell proliferation as was noted with the B cells in the present study.

In conclusion, the inhibition of PP-1 and/or PP-2A in unstimulated B cells results in a rapid increase in AP-1, NF-κB, and TNF-α mRNA. These findings implicates these phosphatases as potent inhibitors of cell activation in B cells and suggests that B cell activation is accompanied by not only an activation of protein kinases but an equally important inhibition of protein phosphastases.

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REFERENCES

1. Kishimoto, T., and Hirano, T. (1989) Paul, W., ed., Raven Press, 385-412.
2. Yamanashi, Y., Kakiuchi, T., Mizuguchi, J., Yamamoto, T., and Toyoshima, K. (1991) Science 251, 192-294.
3. Coggeshall, K.M., and Cambier, J.C. (1985) J. Immunol. 134, 101-107.
4. Valentine, M.A., Meier, K.E., Rossie, S., and Clark, E.A. (1989) J. Biol. Chem. 264, 11282-22387.
5. Tonks, N.K., Charbanneau, H., Diltz, C.D., Fischer, E.H., and Walsh, K.A. (1988) Biochemistry 27, 8695-8698.
6. Koretzky, G.A., Picus, J., Schultz, T., and Weiss, A. (1991) Proc. Natl. Acad. Sci. USA: 88, 2037-2041.
7. Smeland, E.B., Holte, H., Blomhoff, H.K., Asheim, H.C., Stokke, T., Torjesen, P., and Funderud S. (1990) Scand. J. Immunol. 31, 583-591.
8. Cohen, P., and Cohen, P.T.W. (1989) J. Biol. Chem. 254, 21435-21438.
9. Cohen, P., Holmes, C.F.B., and Tsukitani, Y. (1990) Trends Biochem. 15, 98-102.
10. Tachibana, K., Scheuer, P.J., Tsukitani, Y., Kikuchi, H., Van Eugen, D., Clardy, J., Gochipand, H., and Schmitz, F.J. (1981) J. Am. Chem. Soc. 103, 2469-2471.
11. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura, T. (1988) Proc. Natl. Acad. Sci. USA 85, 1768-1771.
12. Bialojan, C., and Takai, A. (1988) Biochem. J. 256, 283-290.
13. Thévenin, C., Kim, S.J., and Kehrl, J.H. (1991) J. Biol. Chem. 266, 9363-9366.
14. Chedid, M., Yoza, B.K., Brooks, J.W., and Mizel, S.B. (1991) J. Immunol. 147, 867-873.
15. Thévenin, C., Kim, S.J., Rieckmann, P., Fujiki, H., Norcross, M.A., Sporn, M.B., Fauci, A.S., and Kehrl, J.H. (1990) New Biol. 2, 793-800.
16. Falkoff, R.J.M., Muraguchi, A., Hong, J.X., Butler, J.L., Dinarello, C.A., and Fauci, A.S. (1983) J. Immunol. 131, 801-806.
17. Osborn, L., Kunkel, S., and Nabel, G.J. (1989) Proc. Natl. Acad. Sci. USA 86, 2336-2340.
18. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729-789.

19. Lenardo, M.J., and Baltimore, D. (1989) Cell 58, 227-228.
20. Staudt, L.M., Clerc, R.G., Singh, H., LeBowitiz, J.H., Sharp, P.A., and Baltimore, D. (1988) Science 241, 577-580.
21. Rieckmann, P., D'Alessandro, F., Nordan, R.P., Fauci, A.S., and Kehrl, J.H. (1991) J. Immunol. 146, 3462-3468.
22. Angel, P., Hattori, K., Smeal, T., and Karin, M. (1988) Cell 55, 875-885.
23. Kehrl, J.H., Thévenin, C., Rieckmann, P., and Fauci, A.S. (1991) J. Immunol. 146, 4016-4023.
24. Bours, V., Villalobos, J., Burd, P.R., Kelly, K., and Siebenlist, U. (1990) Nature 348, 76-80.
25. Roberts, S.B., Segil, N., and Heintz, N. (1991) Science 253, 1022-1026.
26. Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T., and Nishimoto, T. (1990) EMBO J. 9, 4331-4338.
27. Economou, J.S., Rhoades, K., Essner, R., McBride, W.H., Gasson, J.C., and Morton, D.L. (1989) J. Exp. Med. 170, 321-326.
28. Collart, M.A., Baeuerle, P., and Vassalli, P. (1990) Mol. Cell. Biol. 10, 1498-1506.
29. Metcalfe, S., and Milner, J. (1990) Immunol. Lett. 26, 177-182.