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CYCLIC GUANOSINE 3',5'-MONOPHOSPHATE AND PHOSPHODIESTERASE ACTIVITY IN MITOGEN-STIMULATED HUMAN LYMPHOCYTES

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SUMMARY - The cyclic adenosine 3',5'-monophosphate (cyclic AMP) phosphodiesterase from human leukemic lymphocytes differs from the normal cell enzyme in having a much higher activity and a loss of inhibition by cyclic guanosine 3',5'-monophosphate (cyclic GMP). In an effort to determine the mechanism of these alterations, we have studied this enzyme in a model system, lectin-stimulated normal human lymphocytes. Following stimulation of cells with concanavalin A (con A) the enzyme activity gradually becomes altered, until it fully resembles the phosphodiesterase found in leukemic lymphocytes. The changes in the enzyme parallel cell proliferation as measured by increases in thymidine incorporation into DNA. The addition of a guanylate cyclase inhibitor preparation from the bitter melon prevents both the changes in the phosphodiesterase and the thymidine incorporation into DNA. This blockage can be partially reversed by addition of 8-bromo cyclic guanosine 3',5'-monophosphate (8-bromo cyclic GMP) to the con A-stimulated normal lymphocytes. These results indicate a possible role of cyclic GMP in a growth related alteration of cyclic AMP phosphodiesterase.

INTRODUCTION - Cyclic nucleotide phosphodiesterases of animal tissues exist in multiple forms, distinguishable by substrate specificity, sensitivity to certain biological regulators, and subcellular localization (1). Recent reports indicate changes in the expression of these enzymes during conditions of rapid cell growth or transformation. Shoji et al. (2) found that hepatoma cells have decreased cyclic GMP phosphodiesterase activity and an increase in the activity of a low Km cyclic AMP phosphodiesterase, when compared to normal liver cells. Similar differences are found in pheochromocytoma cells (3) and in human mela-

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The abbreviations used are: Cyclic AMP, cyclic adenosine 3',5'-monophosphate; cyclic GMP, cyclic guanosine 3',5'-monophosphate; con A, concanavalin A; 8-bromo cyclic GMP, 8-bromo cyclic guanosine 3',5'-monophosphate.

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nomas (4). Lymphocytes from tumor-bearing AKR mice exhibit increases in the activity of both cyclic AMP and cyclic GMP phosphodiesterases, when compared to cells from non-tumor-bearing AKR mice (5).

We have recently reported that homogenates of human peripheral blood lymphocytes contain a single phosphodiesterase which hydrolyzes only cyclic AMP (6). No cyclic GMP phosphodiesterase activity was detected in homogenates of these cells. The cyclic AMP phosphodiesterase from the normal donors' lymphocytes was 85% inhibited by a 10-fold excess of cyclic GMP, while the enzyme from the leukemic patients' lymphocytes, or from the lymphoblastic lines, was inhibited only 15%. The phosphodiesterase activities from the lymphoblastoid cell lines and from the leukemic lymphocytes were considerably higher than that of the normal lymphocytes.

The induction of cyclic AMP phosphodiesterase by its substrate has been established by a number of investigators. Human diploid fibroblasts, exposed to high cyclic AMP levels, exhibit an increase in the activity of a low Km cyclic AMP phosphodiesterase in a process requiring RNA and protein synthesis (7). Exposure of 3T3 and SV3T3 fibroblasts (8), chick embryo fibroblasts (9), L929 cells (10), and rat astrocytoma cells (11) to high cyclic AMP levels also results in increased cyclic AMP phosphodiesterase activity. Induction of this enzyme by cyclic GMP has not been reported although cyclic GMP levels are increased in lymphoblastoid cell lines (6), and in mitogen-stimulated normal lymphocytes (12).

We have investigated the possible correlation between cyclic GMP and the altered cyclic AMP phosphodiesterase activity observed in these cells using con A-stimulated normal human lymphocytes, a guanylate cyclase inhibitor, and a cyclic GMP analog. Our results suggest that cyclic GMP is involved in the induction of the aberrant cyclic AMP phosphodiesterase in mitogen-stimulated and leukemic human lymphocytes.

METHODS - Purification of Human Peripheral Blood Lymphocytes - Normal human lymphocytes were isolated from venous blood by defibrination and separation on

Ficoll-Hypaque, under sterile conditions (6). Lymphocyte yields were approximately 800 x 10^6 cells per 500 ml of normal blood and cells were greater than 95% viable as judged by trypan blue exclusion. Cell preparations contained less than 1% platelets or red blood cells when isolated in this manner. Each experimental sample was adjusted to contain 100 x 10^6 cells in 30 ml of RPMI 1640 medium (Gibco, Grand Island, N.Y.) plus 20% fetal calf serum.

Mitogen Stimulation of Norman Human Lymphocytes - Normal human lymphocytes, prepared as described above, were exposed to sterile con A (Sigma, 25 µg/ml) and aliquots were taken for analysis at 0,1,4,6,24,48, and 73 hours. Tritiated thymidine incorporation ([methyl-^{3}H] thymidine, 90 Ci/mmole, ICN) into DNA was reported as cpm incorporated into 10% trichloracetic acid precipitable material.

Phosphodiesterase Assay - Cells were harvested by centrifugation, washed twice in phosphate-buffered saline, suspended in 5 ml of 50 mM Tris (pH 7.4), and sonicated (20 seconds at 50 watts, Biosonic IV). Phosphodiesterase activity was assayed according to Thompson and Appleman (13), using a standard incubation mixture of 0.4 ml total volume, containing 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 1 µM cyclic AMP, 40,000 cpm of ^{3}H-labeled cyclic AMP (New England Nuclear, 46.5 Ci/mmole), and 0.2 ml of the sonicated cell preparation. After incubation at 30°C for 10 to 30 minutes, the reaction was terminated by boiling for one minute. The two-step assay was then completed by addition of 0.1 ml of snake venom (Crotalus atrox, 1 mg/ml), incubation for 10 minutes, and removal of residual substrate with 1 ml of a 50% slurry of the anion exchange resin, Biorad AG 1X2 (Chloride form in 50% ethanol.) Aliquots of the supernatant were counted by liquid scintillation. Product recoveries were calculated using [^{3}H] adenosine. Protein concentrations were estimated according to the procedure of Lowry et al. (14) using bovine serum albumin as a standard. Specific activity is reported as picomoles of cyclic AMP hydrolyzed per minute per mg total cell protein.

Preparation of a Crude Guanylate Cyclase Inhibitor - Bitter melon (Momordica charantia abbreviata, also called balsam pear) was obtained at local markets and prepared by a modification of Claflin et al. (15). The whole ripe fruit was homogenized in saline at pH 7.6 and centrifuged for 20 minutes at 12,000 x g. That volume of bitter melon extract which contained 250 µg of protein/ml of incubation medium was determine to have maximal effects on [methyl-^{3}H] thymidine incorporation.

RESULTS - With increasing time of exposure to con A, the specific activity of the cyclic AMP phosphodiesterase in homogenates of human lymphocytes increased from 9.6 units/mg protein to 69 units/mg protein (Figure 1). The increase in specific activity, initially observed at 4 hours, was essentially complete by 24 hours. Concomitant with the rise in specific activity there was a decrease in cyclic GMP inhibition of cyclic AMP hydrolysis (from 56% to 16%). This change was detectable as early as 4 hours after exposure to the mitogen. Thus, the phosphodiesterase activity from these mitogen-stimulated cells rapidly undergoes changes similar to those seen in the enzyme from leukemic or transformed cells (6).

The effects of a guanylate cyclase inhibitor on con A-induced changes in phosphodiesterase are shown in Table 1. The crude bitter melon extract blocked

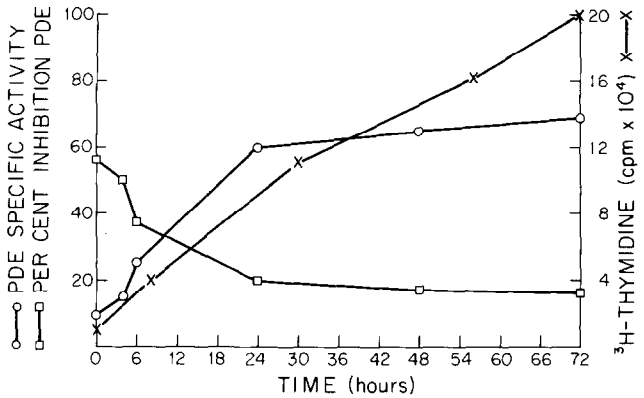

Figure 1 - The effects of mitogen stimulation on normal human lymphocyte phosphodiesterase. Lymphocytes were cultured, as described in methods, with the addition of 25 µg/ml con A. Specific activity of the phosphodiesterase (PDE) (0————0) is expressed as picomoles of cyclic AMP hydrolyzed/minute/mg protein, using 1.0 µM cyclic AMP as substrate. Per cent inhibition (|¯|————|¯|) is expressed as inhibition of the hydrolysis of 1 µM cyclic AMP by 10 µM cyclic GMP. Tritiated thymidine ([methyl-^{3}H] thymidine, 0.015 µCi per ml) incorporation (x————x) is expressed as cpm precipitable with 10% trichloroacetic acid. The results expressed were obtained with cells from a representative single donor.

the con A-induced change in cyclic GMP inhibition of cyclic AMP hydrolysis, the increase in enzyme specific activity, and the increase in thymidine incorporation into DNA. The inclusion of 1 mM 8-bromo cyclic GMP overcame the effect of the melon extract on cyclic GMP inhibition of the phosphodiesterase and on thymidine incorporation, but did not restore the elevated enzyme activity. Parallel experiments indicate that the bitter melon extract as well as the cyclic GMP analog did not affect these parameters in the absence of con A. Eight-bromo cyclic AMP cannot replace the cyclic GMP analog in the reversal of the block of con A effects. The elevation in cyclic GMP levels seems necessary for the con A-induced alterations in phosphodiesterase. The finding that the presence of the cyclic GMP analog could not reverse all the effects of the guanylate cyclase inhibitor nor directly replace con A in inducing mitogenesis suggests that additional factors may influence the expression of these enzymes.

DISCUSSION - Although a role for cyclic AMP in cellular processes has been established, the role of cyclic GMP has been less well defined. Recent results suggest a nuclear site of action for cyclic GMP induction of growth. Spruill

TABLE 1

EFFECTS OF BITTER MELON EXTRACT CONTAINING A GUANYLATE CYCLASE INHIBITOR ON CON A-STIMULATION OF HUMAN LYMPHOCYTES

Sample	PDE Specific Activity [1] (Units/mg protein)	^{3}H Thymidine [2] Incorporation (cpm)	Cyclic GMP Inhibition [3] (per cent)	Viable Cells [4] (% of total)
No Additions	36	16,358	61%	100%
Con A	106	25,448	17%	100%
Con A + Bitter melon extract	30	16,238	60%	98%
Con A + Bitter melon extract + 8-bromo cyclic GMP	26	25,293	27%	94%

[1] Phosphodiesterase (PDE) activity is expressed as picomoles hydrolyzed/minute/mg protein, using 1.0 µM cyclic AMP as substrate. All samples were measured after 48 hours of incubation.

[2] Tritiated thymidine ([methyl-^{3}H] thymidine 90 Ci/mmole, ICN) incorporation was measured using 0.5 µCi, added at zero time.

[3] Cyclic GMP inhibition was measured using 1.0 µM cyclic GMP as inhibitor.

[4] Cell viability was measured by trypan blue exclusion.

[5] Final incubation concentrations were: Con A 25 µg/ml. Bitter melon extract (Momordica charantia abbreviata) 250 µg protein per ml of medium; 8-bromo cyclic GMP 1.0 mM. All additions were made at zero time. These results are from cells of one human donor, assayed in triplicate. Other donors showed the same effects but absolute values varied by up to 150%

et al. (16) demonstrated that cyclic GMP but not cyclic AMP was localized on puffs of polytene chromosomes of Drosophila melanogaster and, after heat shock, this association was maintained. The localization correlated with that of RNA polymerase II. Studies of the regenerating liver system have shown that cyclic GMP and guanylate cyclase activity increase in nuclear membranes and intranuclear regions (17). Furthermore, Johnson et al. (18) reported that cyclic GMP enchances DNA binding to histones and one specific non-histone nuclear protein. Cyclic GMP levels are reported to increase dramatically during phytohemagglutinin-induced lymphocyte proliferation (12). To establish if a correlation exists between the early elevation in cyclic GMP and the later changes in phosphodiesterase, we have used a con A-stimulated normal human lymphocyte system. The experiments reported here show that within four hours of con-A stimulation, the phosphodiesterase activity of normal lymphocytes changes to match that of the transformed cells. This accompanied an increase in thymidine incorporatin into DNA. The alteration in the enzyme activity appears to require an increase in cyclic GMP levels since addition of a guanylate cyclase inhibitor blocks the change in the enzyme and the block can be partially reversed by addition of the analog, 8-bromo cyclic GMP. The fact that 8-bromo cyclic GMP only partially reversed the block by the guanylate cyclase inhibitor may indicate that the changes in inhibition by cyclic GMP, and the increase in specific activity are not directly related phenomena. The presence, in the crude bitter melon preparation, of other unknown effectors acting at other sites is also possible.

While the specific sites of action have yet to be elucidated, it is becoming clear that cyclic GMP can play a key role in the regulation of growth at the cellular level. The information presented here and from other laboratories suggests a role for cyclic nucleotides during mitogenic transformation. An early step in the process would be the elevation of cyclic GMP levels, probably through the increased activity of guanylate cyclase. Any increase in the levels of cyclic GMP could be amplified in some cells by the disappearance of those forms

of the phosphodiesterase which can hydrolyze it. In lymphocytes, the cyclic GMP would induce a change in cyclic AMP phosphodiesterase to a form which is more active and less susceptible to inhibition by cyclic GMP. The resulting increase in cyclic AMP hydrolysis would contribute to the reciprocal changes in these two cyclic nucleotides which are seen both during mitogenic transformation and during malignancy.

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