Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 25-30

INTERFERENCE OF RETINOIC ACID BINDING TO ITS BINDING PROTEIN BY OMEGA-6 FATTY ACIDS

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Received July 6, 1987

Cellular retinoic acid-binding protein (CRABP) is the putative mediator of the biological effects of retinoic acid in the control of epithelial differentiation and tumorigenesis. Omega-6 fatty acids such as linoleic acid and arachidonic acid, precursors of prostaglandin synthesis, caused inhibition of retinoic acid binding to CRABP. These fatty acids, however, possessed lower affinity than retinoic acid for the binding protein. Omega-3 fatty acids, such as eicosapentaenoic acid and docosohexaenoic acid, did not cause such inhibition in the binding of retinoic acid. Whereas retinoic acid was a potent modulator of differentiation of F9 embryonal carcinoma cells, neither omega-3 nor omega-6 fatty acids showed any significant differentiation potential. Competition by omega-6 fatty acids with retinoic acid for CRABP may neutralize the binding protein-mediated biological functions of retinoic acid, and could thereby enhance tumor production.

Retinoids can prevent or reverse the process of carcinogenesis (1). Retinoic acid, an oxidized form of retinol, has a greater potency than retinol in the control of differentiation and tumorigenesis (2-4). A major theory to explain the mode of action of retinoids in the control of epithelial differentiation and tumorigenesis centers around cellular retinolbinding protein (CRBP) and cellular retinoic acid-binding protein (CRABP). Although the basic concept of the genomic control mechanism of retinoic acid is not yet clear, it appears to involve migration of the retinoic acid-CRABP complex from the cell membrane through the cytoplasm to the nucleus where it exerts a direct effect upon gene expression (5).

Recently the role of polyunsaturated fatty acids (PUFA), especially omega-3 versus omega-6 types, in neoplastic disease has received much attention. Cancer incidence is low in Eskimo populations whose fish-rich diet is high in omega-3 PUFA, principally, α-linolenic acid, eicosapentaenoic acid, and docosohexaenoic acid (8,9). In contrast, omega-6 fatty acids, principally linoleic acid and arachidonic acid, promote

Abbreviations: CRABP, cellular retinoic acid-binding protein; PUFA, polyunsaturated fatty acids.

0006-291X/87 $1.50

the development of tumors (9-11). The mechanism by which PUFA affect the carcinogenic process is not clearly understood although formation of lipid peroxides and their decomposition products, such as free radicals, appears to be involved in these processes (12). Additionally, linoleic acid and arachidonic acid are precursors for biologically active prostaglandins, such as PGE_2 which in excess are immunosuppresive and hence tumor promoting (13).

Since retinoids are effective inhibitors of carcinogenesis and omega-6 PUFA are tumor promoters, it was important to investigate whether there is any meaningful interrelationship between their modes of action at the molecular level. The biological function of retinoic acid is believed to be mediated by its binding protein, CRABP (5-7). We now report that two prominent omega-6 PUFA, arachidonic acid and linoleic acid, compete with retinoic acid for its specific binding sites on CRABP. The omega-3 PUFA do not exhibit such an inhibition.

MATERIALS AND METHODS

Retinoic acid and 11,12[^{3}H]retinoic acid (1.28 Ci/mmol) were supplied by the National Cancer Institute, Bethesda, MD. Fatty acids, plasminogen, fibrinogen, and H-D-Val-Leu-Lys-p-nitroaniline were purchased from Sigma Chemical Co., St. Louis, MO.

Binding affinities of retinoids and PUFA for CRABP were determined by the sucrose density gradient sedimentation technique (14). Extracts from 12- to 13-day old chick embryo skins were passed through Affi-Gel Blue column to remove traces of serum albumin (14). Aliquots (2 mg protein) of these preparations were incubated with 300 pmoles of [^{3}H]retinoic acid in the presence or absence of a 100-fold molar excess of the test compounds. The 2S CRABP peaks were determined from the radioactivity profiles obtained after sedimentation through 5 to 20% sucrose density gradients at 180,000 x g for 18 hr. The inhbition of [^{3}H]retinoic acid binding caused by a 100-fold molar excess of unlabeled retinoic acid is regarded as 100% inhibition. Inhibition caused by a similar excess of test compounds is expressed as relative inhibition to the above standard.

In order to calculate the concentrations of retinoids and PUFA that produce 50% inhibition (I_{50}) of binding of [^{3}H]retinoic acid to CRABP, portions of chick skin extracts (1 mg protein) were incubated with 100 pmoles of [^{3}H]retinoic acid in the presence or absence of 1-, 5-, 10- and 25-fold molar excesses of unlabeled test compounds. Free radioactive retinoid was removed by absorption on dextran-coated charcoal. The I_{50} values of the test compounds were derived from semilog plots of their molar concentrations versus the specifically bound [^{3}H]retinoic acid to CRABP at different concentrations of the test compounds.

Evaluation of the differentiation potential of retinoic acid and PUFA was carried out using an embryonal carcinoma cell line, F9 (15). Elevation of plasminogen activator release by F9 cells in the presence of retinoids is regarded as a marker for inducing differentiation into parietal endoderm (15). F9 cells were grown at a density of 1 x 10^5 cells/ml in the presence or absence of 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11}M retinoic acid or PUFA for 4 days. Aliquots (20 µl) of the harvest fluid were mixed with 0.13 µm plasminogen, 0.3 mM H-D-Val-Leu-Lys-p-NA (a synthetic substrate of plasmin), 0.1% Tween 80, and 25 µg of fibrinogen fragments in a final volume of 0.2 ml of Tris-HCl, pH 7.5 (16). After incubation or 4 hr at 25°C, the generation of p-nitroaniline was measured by absorbance at 405 nm. The mid-point between the maximal and minimal

absorbance values of a test compound is defined as the ED_{50} concentration of that compound.

RESULTS AND DISCUSSION

The effect of competition of fatty acids against [^{3}H]retinoic acid for binding to CRABP are shown in Fig 1. The sucrose density gradient profiles reveal that a 100-fold molar excess of arachidonic acid competed quite efficiently for retinoic acid-binding sites on CRABP. Linoleic acid showed inhibition to a lesser extent, but oleic acid was without any significant effect on the binding of [^{3}H]retinoic acid. The omega-3 fatty acids, eicosapentaenoic acid and docosohexaenoic acid did not produce competition against retinoic acid for CRABP (not shown). Thus the results indicate that omega-6-PUFA, but not omega-3-PUFA, can interfere with the binding of [^{3}H]retinoic acid to CRABP.

Table 1 illustrates the relative binding affinities of several randomly selected fatty acids for CRABP. Saturated fatty acids containing up to 18 carbon atoms did not exhibit any binding affinity for CRABP. Among the unsaturated fatty acids that were examined, linoleic acid and arachidonic acid showed 50% and 85%, respectively, binding affinity for CRABP as compared to 100% for retinoic acid. Our earlier results indicate that γ-linolenic acid (6,9,12-octadecatrienoic acid), another omega-6 fatty acid, does not compete with [^{3}H]retinoic acid for CRABP (17). Thus it may not be generalized that all omega-6 fatty acids exhibit affinity for CRABP. Certain specific

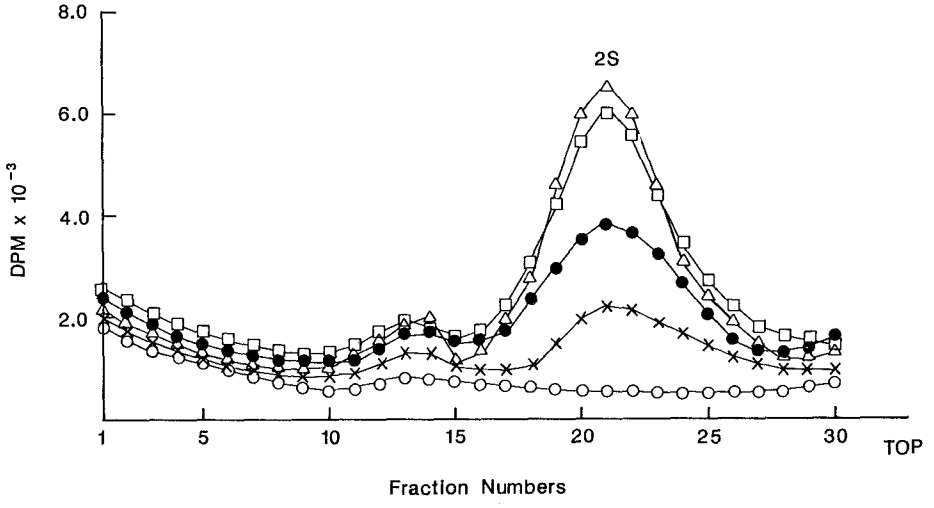

Fig. 1. Sucrose density gradient pattern showing the effect of competion of unlabeled test compounds on the binding of [^{3}H]retinoic acid to chick embryo skin CRABP. Δ, control with 300 pmoles of [^{3}H]retinoic acid. All the other radioactivity profiles are the control plus 100-fold molar excesses of the following test compounds: O, retinoic acid; X, arachidonic acid; ●, linoleic acid; □, oleic acid.

TABLE 1

PERCENTAGE INHIBITION OF BINDING OF [^{3}H]RETINOIC ACID TO CRABP BY 100-FOLD MOLAR EXCESSES OF UNLABELED TEST COMPOUNDS

Fatty Acids-Retinoic Acid			
Number of C Atoms	Common Name	Systematic Name	Inhibition(%)
2	Acetic	-	0
3	Propionic	-	0
4	Butyric	-	0
6	Caproic	n-hexanoic	0
8	Caprylic	n-octanoic	0
12	Lauric	n-Dodecanoic	0
16	Palmitic	n-Hexadecanoic	0
18	Stearic	n-Octadecanoic	0
18	Oleic	cis-9-Octadecanoic	5
18	Linoleic	cis-cis-9-12-Octadecadienoic	50
20	Arachidonic	5,8,11,14-Eicosatetraenoic	85
20	-	5,8,11,14,17-Eicosapentaenoic	10
22	-	4,7,10,13,16 19-Docosohexaenoic	0
20	Retinoic acid	All-trans-retinoic acid	100

structural features are required for competition. Although structurally dissimilar in certain aspects, both arachidonic acid and retinoic acid contain 20 carbon atoms. Also, like arachidonic acid, retinoic acid has 4 carbon-carbon double bonds in the side chain, which, however, are positioned in allyl units in the former but in a conjugated arrangement in the latter. On the contrary, eicosapentaenoic acid with 20 carbon atoms and 5 double bonds, and docosohexaenoic acid with 22 carbon atoms and 6 double bonds exhibited little or no competition with retinoic acid, presumably because of the presence of the omega-3 double bond. It is of interest that retinoic acid itself can be visualized as a modified omega-6 acid, if one of the carbon atoms of the gem dimethyl carbons in the cyclohexenyl ring is designated as the omega-1 carbon and that fusion has occurred between the omega-2 and omega-7 carbons.

Retinoic acid is a well known anticarcinogenic agent and modulator of epithelial differentiation (1-4). Since arachidonic acid and linoleic acid compete with retinoic acid for CRABP, it was important to investigate whether these fatty acids, like retinoic acid, can induce differentiation of F9 cells. Table 2 illustrates the ED_{50} values of retinoic acid and PUFA on plasminogen activator release from F9 cells, and the I_{50} values on the inhibition of retinoic acid-binding to CRABP. The ED_{50} value of retinoic acid for inducing differentiation was 1×10^{-10}M, and the I_{50} for displacement of [^{3}H]retinoic acid from CRABP was 5×10^{-7}M. For arachidonic acid and linoleic acid, the I_{50} values were 4×10^{-6}M and 2.5×10^{-5}M, respectively, and for oleic acid, eicosapentaenoic acid and docosohexaenoic acid, these values were $>1 \times 10^{-4}$M. Whereas retinoic acid expressed pronounced activity (ED_{50}, 1×10^{-10}M) in the induction of differentiation in F9 cells, both omega-3 and omega-6 fatty acids were poor inducers of differentiation with ED_{50} values 2-3 $\log_{10}$ lower than that of retinoic acid.

TABLE 2

ED_{50} AND I_{50} VALUES OF FATTY ACIDS AND RETINOID ACID FOR RELEASE OF PLASMINOGEN ACTIVATOR AND FOR INHIBITION OF [^{3}H]RETINOIC ACID-BINDING TO CRABP

Fatty Acids/Retinoic Acid	ED_{50} Values (Plasminogen Activator Release from F9 Cells)	I_{50} Values (Inhibition of [^{3}H]Retinoic Acid-Binding to CRABP)
Retinoic Acid	1×10^{-10}M	5×10^{-7}M
Arachidonic Acid	2×10^{-8}M	4×10^{-6}M
Linoleic Acid	8×10^{-8}M	2.5×10^{-5}M
Oleic Acid	$>1 \times 10^{-7}$M	$>1 \times 10^{-4}$M
Eicosapentaenoic Acid	$>1 \times 10^{-7}$M	$>1 \times 10^{-4}$M
Docosohexaenoic Acid	$>1 \times 10^{-7}$M	$>1 \times 10^{-4}$M

The results of our studies clearly indicate that omega-6 fatty acids are interfering with the interaction of retinoic acid with CRABP. Because the binding affinities of these fatty acids are lower than that of retinoic acid for CRABP, retinoic acid may be considered as the natural and preferred ligand of the binding protein within the cell. Even though the present data suggests that omega-6 fatty acids displace [^{3}H]retinoic acid from CRABP, binding studies using radiolabeled fatty acids will be required to prove their actual binding at retinoic acid-binding sties on CRABP. However, we have now illustrated an interrelationship at molecular level between two groups of compounds with opposite actions. It may be assumed that by inhibiting the retinoic acid binding to CRABP, both arachidonic acid and linoleic acid are nullifying the CRABP-mediated functions of retinoic acid in the control of differentiation and tumorigenesis. Omega-3 fatty acids do not show such inhibition. It has been postulated that they control tumor production by inhibiting prostaglandin synthesis (13).

Linoleic acid is the obligatory precursor of arachidonic acid, the principal substrate of prostaglandin synthesis (18). Prostaglandins play significant regulatory roles in the homeostasis of several organ and cell signal systems. A CRABP-mediated role of arachidonic acid in the synthesis of prostaglandins is highly speculative but cannot be excluded. We have, however, shown earlier that prostaglandins do not exhibit binding affinity for CRABP (19). A detailed study on the interaction of omega-3 and omega-6 fatty acids in relation to retinoic acid-CRABP interactions may contribute to a clearer understanding of the interrelationship between the biological actions of retinoids and fatty acids.

ACKNOWLEDGEMENTS

This work was supported by Grant CA 40756 from the National Cancer Institute, Bethesda, MD. We thank Drs. Robert F. Struck and Dennis J. McCarthy of our Institute for useful discussions.

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