EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS AND ANTIOXIDANTS ON THE CLONAL GROWTH OF HUMAN DIPLOID FIBROBLASTS

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INTRODUCTION

Although exogenous prostaglandins have potent effects on the multiplication of a variety of cell types, endogenous prostaglandin biosynthesis has not been shown to be required for multiplication of human diploid cells *in vitro*.[1,2,4] Likewise, linoleic acid (EFA), or one of its metabolites, has not been proven to be an essential nutrient for the clonal growth of human diploid cells. Linoleic acid is an essential nutrient for mammals and its essentiality may be due to its role as a precursor for prostaglandin biosynthesis.[5]

What role do prostaglandins and other EFA metabolites play in the regulation of multiplication of human diploid cells? These experiments were designed to examine the effects of non-steroidal anti-inflammatory agents (NSAIA), which inhibit prostaglandin biosynthesis, and antioxidants, which interact with EFA metabolism in several pathways, on the clonal growth of human diploid fibroblasts. Additional experiments were performed to measure the effects of these agents on the clonal growth of human diploid cells in the presence of exogenous prostaglandins.

MATERIALS AND METHODS

Materials

$PGF_{2\alpha}$, PGD_2, thromboxane B_2 (TXB_2), PGE_2, PGE_1 and ibuprofen were donated by the Upjohn Company. 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) was a gift from Unilever Research. PGA_1, PGA_2, PGB_1, PGB_2, $PGF_{1\alpha}$, indomethacin, aspirin, salicylate, phenylbutazone, butylated hydroxyanisole, butylated hydroxytoluene and propyl gallate were purchased from Sigma Chemical Co. Flufenamic acid was purchased from Aldrich Chemical Co., ethoxyquin from Pfaltz and Bauer, Inc. and N,N′-diphenyl-p-phenylenediamine from Fisher Scientific Co.

Culture Media

MCDB 107 is a modification of MCDB 104[3] with the following quantitative changes: glycine 3×10^{-4} M, KCl 5×10^{-3} M, NaCl 1.1×10^{-3} M, HEPES 3×10^{-2} M, NaOH approximately 2×10^{-2} (pH in air at room temperature 7.6). It is designed for use with 2% CO_2. Dialyzed fetal bovine serum protein (FBSP) is used as a media supplement.[3]

Assay Methods

All tissue culture methods for the maintenance of human diploid fibroblasts (HDF) were previously described.[3] Flow 2000 human diploid fibroblasts (passage 13) were inoculated at a density of 200 cells/60 mm plate (polylysine-coated) and grown in 5 ml of medium MCDB 107 containing 200 μg/ml FBSP. After 14 days, cells were fixed, stained

TABLE 1. Effects of Non-Steroidal Anti-Inflammatory Agents and Antioxidants on the Clonal Growth of Human Diploid Fibroblasts

Treatment	Relative growth[1]	Treatment	Relative growth
Control	100%	D-α-tocopherol	
		(10^{-8} M)	145%*
		(10^{-5} M)	50%
Control and vehicle[2]	100%	D-α-tocopherol acetate	
		(10^{-7} M)	150%*
		(10^{-5} M)	110%
Indomethacin		*n*-propyl gallate	
(10^{-10} M)[3]	165%*	(10^{-8} M)	155%*
(10^{-5} M)	95%	(10^{-5} M)	5%
Flufenamic Acid		Butylated hydroxy toluene	
(10^{-8} M)	155%*	(10^{-8} M)	145%*
(10^{-5} M)	110%	(10^{-5} M)	0%
Ibuprofen		Butylated hydroxy anisole	
(10^{-8} M)	165%*	(10^{-10} M)	100%
(10^{-5} M)	100%	(10^{-5} M)	0%
Phenylbutazone		Ethoxyquin	
(10^{-7} M)	145%*	(10^{-10} M)	100%
(10^{-5} M)	120%	(10^{-5} M)	0%
Aspirin		N-N′-diephenyl-p-phenylene-diamine	
(10^{-6} M)	150%*	(10^{-10} M)	100%
(10^{-5} M)	120%	(10^{-5} M)	0%
Salicylate			
(10^{-4} M)	155%*		

[1]Values indicated by (*) are significantly higher than controls by the student's *t*-test ($P < 0.05$).
[2]Vehicle consists of ethanol at a final concentration of 0.1%.
[3]The first concentration listed for each agent is the concentration that gives maximal stimulation.

with crystal violet and total colony area determined with an Artek model 880 colony counter. Test compounds were dissolved in absolute ethanol and were added to the assay media resulting in a final concentration of 0.1% ethanol.

RESULTS AND DISCUSSION

A variety of NSAIA significantly stimulate the clonal growth of Flow 2000 human diploid fibroblasts grown in MCDB 107 plus 200 μg/ml FBSP. The results are shown in Table 1. Each NSAIA exhibits a characteristic dose response curve that correlates with the reported ability of the compound to inhibit prostaglandin biosynthesis and prevent experimentally induced inflammation. Thus, in terms of specific activity, indomethacin > ibuprofen ≈ flufenamate > phenylbutazone > aspirin > salicylate. The apparent lack of toxicity of NSAIA on the clonal growth of HDF suggests a minimal involvement of endogenous prostaglandin biosynthesis in the multiplication process of HDF.

Several antioxidants, including α-tocopherol, α-tocopherol acetate, *n*-propyl gallate and butylated hydroxytoluene, significantly stimulate the clonal growth of HDF at low FBSP levels when present in the concentration range of 10^{-8} to 10^{-7} M. The antioxidants butylated hydroxyanisole, ethoxyquin and N,N′-diphenyl-p-phenylenediamine do not stimulate growth at any concentration apparently due to a stronger toxic effect. All the antioxidants, except α-tocopherol acetate, inhibit the clonal growth of HDF at concentrations $\geq 10^{-6}$ M. The toxic effect of a wide variety of antioxidants could be due to a physical disruption of the cell membranes or, more interestingly, by inhibition of a specific metabolic pathway vital to cell multiplication in HDF.

Prostaglandins added to the culture medium have dramatic effects on the clonal growth of HDF which conveniently fall into two categories (Table 2). Type I prostaglandins, including $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGB_2 and PGB_1, do not affect clonal growth in the physiological range ($10^{-10} - 10^{-8}$ M) but significantly improve growth at pharmacological levels (10^{-5} M). Type II prostaglandins, including PGE_2, PGE_1, PGA_2, PGA_1 and

TABLE 2. Effects of Prostaglandins and Related Compounds on the Clonal Growth of Human Diploid Fibroblasts

Treatment	Relative growth[1]	Treatment	Relative growth
Control	100%	TXB_2	
		(1×10^{-5} M)	100%
		(1×10^{-9} M)	100%
Control and vehicle[2]	100%	HETE	
		(1×10^{-5} M)	95%
		(1×10^{-9} M)	100%
$PGF_{2\alpha}$		Arachidonic acid	
(1×10^{-5} M)	220%*	(1×10^{-5} M)	5%
(1×10^{-9} M)	100%	(1×10^{-9} M)	100%
$PGF_{1\alpha}$		$PGF_{2\alpha}$ (10^{-5} M) +	
(1×10^{-5} M)	170%*	PGE_1 (10^{-5} M)	30%
(1×10^{-9} M)	100%	$PGF_{2\alpha}$ (10^{-5} M) +	
PGB_2		Indomethacin (10^{-10} M)	230%*
(1×10^{-5} M)	200%*	$PGF_{2\alpha}$ (10^{-5} M) +	
(1×10^{-9} M)	100%	Vitamin E acetate (10^{-7} M)	250%*
PGB_1		PGE_1 (10^{-5} M) +	
(1×10^{-5} M)	190%*	Indomethacin (10^{-10} M)	0%
(1×10^{-9} M)	100%	PGE_1 (10^{-5} M) +	
PGE_2		Vitamin E acetate (10^{-7} M)	5%
(1×10^{-5} M)	0%		
(1×10^{-9} M)	160%*		
PGE_1			
(1×10^{-5} M)	0%		
(1×10^{-9} M)	180%*		
PGA_1			
(1×10^{-5} M)	0%		
(1×10^{-9} M)	140%*		
PGA_2			
(1×10^{-5} M)	0%		
(1×10^{-9} M)	145%*		
PGD_2			
(1×10^{-5} M)	0%		
(1×10^{-9} M)	110%		

[1]Values indicated by (*) are significantly higher than controls by the student's t-test ($P < 0.05$).
[2]Vehicle consists of ethanol at a final concentration of 0.1%.

PGD_2 all inhibit the clonal growth of HDF at pharmacological concentrations, however, at physiological levels, PGE_1, PGE_2, PGA_1 and PGA_2 all significantly stimulate clonal growth. These effects suggest that there are two types of prostaglandin receptors in HDF which account for the two types of activity. TXB_2 and HETE both have a minimal effect on clonal growth. However, arachidonic acid is inhibitory at concentrations $\geq 10^{-6}$ M. NSAIA or antioxidants do not appear to interact with the effect of exogenous prostaglandins on clonal growth of HDF.

In summary, NSAIA, which inhibit prostaglandin biosynthesis, stimulate clonal growth of HDF grown in MCDB 107 and 200 μg/ml FBSP. Some antioxidants, including the naturally occurring vitamin E, stimulate clonal growth of HDF; however, all the tested antioxidants are potent inhibitors of clonal growth at surprisingly low concentration. Prostaglandins are potent effectors of clonal growth and appear to act independently of NSAIA and antioxidants. These data do not prove that prostaglandin biosynthesis is a required process for cell multiplication. However, the data do suggest that EFA metabolism, vitamin E status and prostaglandin biosynthesis can all contribute to the regulation of cell multiplication in HDF.

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