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β-Carotene-mediated Inhibition of a DNA Adduct Induced by 7,12-Dimethylbenz(a)anthracene and 7-Hydroxymethyl-12-methylbenz(a)anthracene in Mouse Mammary Gland *in vitro*

Sanjoy K. Das, Ting Z. Jia, Abhik M. Bandyopadhyay and Mihir R. Banerjee

The influence of β-carotene on the formation of DNA-adducts induced by 7,12-dimethylbenz(a)anthracene (DMBA) and 7-hydroxymethyl-12-methylbenz(a)anthracene (7-OHM-12-MBA) during transformation of mouse mammary cells in organ culture was analysed. Treatment with β-carotene (10^{-8}–10^{-5} mol/l) caused inhibition (48.8–94.4%) of an adduct (VI), which was detectable in DNA samples from DMBA-treated mammary glands. Out of six adducts, derived from further analysis of DNA samples from 7-OHM-12-MBA-treated glands, adduct f eluted in the same fraction as adduct (VI), indicating these adducts were analogous. Likewise, adduct f was also inhibited by β-carotene. Boronate chromatographic analysis revealed this particular adduct was a syn-dihydrodiol epoxide product. Adduct inhibition was detectable both at the start and after DMBA treatment. α-Tocopherol and canthaxanthin were ineffective in inhibiting adducts. It is reasonable to conclude that β-carotene-mediated modification of adducts is associated with the inhibition of a syn-adduct, which is derived from further metabolism of a 7-OHM-12-MBA intermediate.
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INTRODUCTION

EPIDEMIOLOGICAL STUDIES have indicated that higher levels of dietary β-carotene and other carotenoids may reduce the tumour incidence in human populations [1]. Although the association between high levels of β-carotene in the diet and reduced tumour incidence may not be causally related, β-carotene may provide a protective influence against tumour incidence [2]. In contrast to the extensive studies [3] on the chemoprevention properties of the compounds having vitamin A-like activity, little is known about the mechanism of the preventive action of β-carotene against the neoplastic disease. There has been interest in elucidating the mechanism by which dietary β-carotene may exert such a protective action [4]. A recent study on biological activities of α-carotene has indicated that when human neuroblastoma cells GOTO were exposed to α-carotene, they were arrested in G_0–G_1 phase of their cell cycle [5]. Earlier, we reported that β-carotene can inhibit 7, 12-dimethylbenz (a) anthracene (DMBA)-induced mammary cell transformation both at the initiation and the promotion stages of carcinogenesis in organ culture of the whole mammary gland in a hormonally-defined serum-free medium [6]. This inhibitory effect is likely to be due to the action of β-carotene itself, since no retinol was measurable in the glands in culture after β-carotene treatment [6]. β-carotene also modifies carcinogen-induced DNA damage and

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reduces sister chromatid exchange in mouse mammary cells which are significant events during the initiation stage of carcinogenesis [7].

Studies on DMBA–DNA adducts from mammalian cells and tissues have indicated that the metabolic activation for DMBA in these systems primarily involves the bay-region dihydrodiol epoxide route [8]. Sawicki *et al.* [9] have shown that both syn- and anti-bay-region dihydrodiol epoxides are major contributors for the binding of DMBA to DNA. The strong mutagenic and carcinogenic activity of DMBA *trans* 3,4-diol [10] and the moderate levels of activities of several monofluoro derivatives in the A-ring of DMBA also suggest the above hypothesis [11]. Although evidence indicates that a dihydrodiol epoxide formed in the A-ring of DMBA is an ultimate carcinogenic metabolite, the high carcinogenic potency of the *trans* 3,4-dihydro-3,4-dihydroxy 7-hydroxymethyl-12-methylbenz(a)anthracene also suggests that the dihydrodiol epoxide formed from this metabolite could be instrumental in the carcinogenic action of DMBA [12]. This concept is further supported by metabolism studies of hydroxymethylbenz(a)anthracene indicating that 3,4 dihydrodiol metabolites are formed and subsequently converted to mutagenic and DNA-binding derivatives [13]. In this report, we present the results of a reverse phase high performance liquid chromatography (HPLC) analysis [14, 15] showing that β-carotene can inhibit a DNA adduct, which is derived from DMBA through an intermediate 7-hydroxymethyl-12-methylbenz (a) anthracene (7-OHM-12-MBA).

MATERIALS AND METHODS

Materials

7,12-[G-^{3}H]dimethylbenz(a)anthracene (specific radioactivity, 1.76 TBq/mmol) was obtained from Amersham. The purity was at least 95–97%. Unlabelled DMBA, β-carotene (from carrot, type IV), fungal protease (type XI), RNase, DNase I, phosphodiesterase I, alkaline phosphatase, α-tocopherol (acid succinate), insulin, aldosterone, hydrocortisone, oestradiol-17β and progesterone were obtained from Sigma. Prolactin and canthaxanthin were a gift from National Hormone and Pituitary Program, NCI, and Hoffmann–La Roche, respectively. Waymouth's medium (MB 752/1) was purchased from Gibco. [^{3}H]-7-OHM-12-MBA (specific radioactivity, 377 GBq/mmol) and [^{3}H]DMBA *trans* 3,4-diol (specific radioactivity 233 GBq/mmol) were prepared by bioconversion of [^{3}H]DMBA in mammary organ culture and subsequent formic acid digestion and purification through a HPLC column [16]. [^{3}H]-7-OHM-12-MBA and [^{3}H]DMBA *trans* 3,4-diol were collected between the retention times 37.0–41.0 min and 24.5–28.5 min respectively from several HPLC runs. Compounds were then dried separately under a slow stream of N_2. The residues were then redissolved in dimethylsulphoxide (DMSO) for use in culture. DMSO concentration never exceeded 0.1% in the culture medium.

Organ culture and treatment with polycyclic aromatic hydrocarbons (PAH) and different chemopreventive agents

The *in vitro* transformation model of the mouse mammary cells in organ culture has been described [17]. Briefly, 3 to 4-week-old BALB/c female mice were primed by daily subcutaneous injections of a mixture of 1 μg oestradiol-17β and 1 mg progesterone for 9 days. On the 10th day, animals were killed by cervical dislocation. All second thoracic mammary glands were excised on sterile dacron rafts and the glands resting on the rafts were then incubated for 6 days in a 95% O_2 and 5% CO_2 atmosphere at 37°C in Waymouth's medium (MB 752/1) supplemented with the mitogenic hormones, insulin, prolactin, hydrocortisone (5 μg/ml each) and aldosterone (1 μg/ml). This hormone mixture induces full lobuloalveolar development of glands *in vitro*.

Consistent with protocol of the transformation experiments, mammary glands were exposed to a transforming dosage of [^{3}H]DMBA (7.8 μmol/l, 740 KBq/ml) or [^{3}H]-7-OHM-12-MBA (11.6 μmol/l, 740 KBq/ml) in 0.1% DMSO (DMSO was used as a vehicle for both PAH carcinogens) for 24 h between the 3rd and 4th day of culture in the mitogenic hormone medium. This treatment induces neoplastic transformation of the mammary epithelial cells [18].

Treatment of β-carotene (10^{-8}–10^{-5} mol/l) was done in *n*-hexane solution with gentle shaking. Concentration of *n*-hexane in the medium was 0.1%. Influence of β-carotene was monitored during the initiation and postDMBA treatment periods. Total initiation period was defined as the 24 h DMBA treatment between 3rd and the 4th day and 3 h additional incubation in DMBA-free medium: total time period being 27 h. α-Tocopherol (10^{-7}–10^{-5} mol/l) and canthaxanthin (10^{-7}–5×10^{-6} mol/l) were also used as different chemopreventive agents. Absolute ethyl alcohol and *n*-hexane were used as vehicles for these compounds, respectively.

DNA isolation and analysis of DNA adducts

DNA was isolated from 1 g of cultured glands by proteinase K and RNase A treatment followed by extraction with chloroform:isoamyl alcohol (24:1) and ethanol precipitation, as described previously [14]. Bound DMBA per mg DNA was measured by determining the content of DNA at $A_{260\ nm}$ and the radioactivity of [^{3}H]DMBA by liquid scintillation spectrometry.

DNA samples were digested enzymatically to deoxyribonucleosides by sequential addition of DNase I (from bovine pancreas), snake venom phosphodiesterase (from *Crotalux atrox* venom), and alkaline phosphatase (from *Escherichia coli*). Modified deoxyribonucleosides were purified by Sep-Pak C_{18} cartridge (Water Associates) after washing with water and 40% methanol and finally the adducts were eluted with 100% methanol (4 ml). Adducts were analysed by HPLC on an ultrasphere ODS column (4.6 mm × 25 cm, Beckman). The column was eluted with a methanol:water gradient system as described previously [14]. Radioactivity was determined by liquid scintillation spectrometry.

Boronate chromatography

Deoxyribonucleoside adducts containing *cis*-vicinal hydroxyl groups (anti-adducts) were separated from other adducts (those that do not contain *cis*-vicinal hydroxyl groups, syn-adducts) by immobilised boronate chromatography using Servacel DHB (Accurate Chemical and Scientific Corp. as described [9]. Syn-adducts were eluted with 1 mol/l morpholine buffer at pH 9.0. Anti-adducts were then eluted with 10% sorbitol solution in 1 mol/l morpholine buffer at pH 9.0. The morpholine buffer fractions and sorbitol–morpholine buffer fractions were pooled for syn- and anti-adducts, respectively and concentrated through a Sep-Pak C_{18} cartridge. Individual adducts were then analysed by reverse-phase HPLC as described [14].

RESULTS

β-carotene influence on PAH-induced DNA adducts formation

Earlier we have reported that mouse mammary glands exposed to DMBA (7.8 μmol/l) for 24 h between the 3rd and 4th day of culture form three major adducts e.g. anti-DMBA-diol-epoxide-

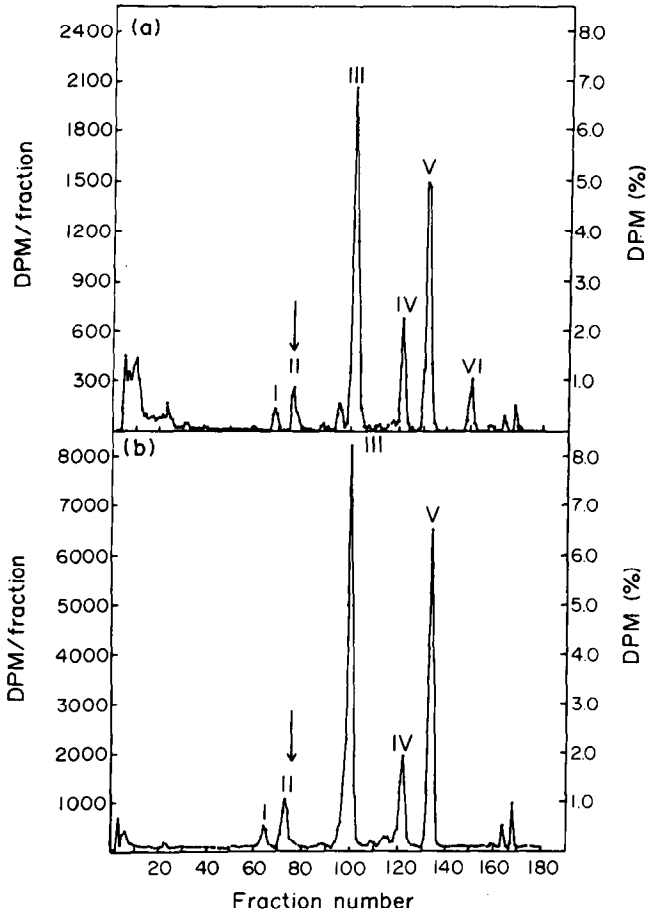

Fig. 1. Effect of β-carotene on the covalent binding of PAH to DNA in mammary glands *in vitro*. β-carotene was treated together with DMBA (a) or 7-OHM-12-MBA (b) for 24 h between 3rd–4th day in culture. Data are the average of triplicate or quadruplicate sets of experiments.

deoxyguanosine (III), syn-DMBA-diol-epoxide-deoxyadenosine (IV) and anti-DMBA-diol-epoxide-deoxyadenosine (V) and three or more minor uncharacterised adducts [14]. β-carotene-treated glands showed an inhibition of adduct (VI) eluting around 60% methanol: retention time 98–101 min, fraction No. 147–152 (Fig. 1). However, the levels of the remaining DMBA–DNA adducts I–V remained unaltered in the presence of β-carotene. In analysis of DNA samples obtained from [^{3}H]-7-OHM-12-MBA-treated mammary glands, 6 DNA-adduct peaks were consistently observed in HPLC chromatogram (Fig. 2a, peaks a to f). DNA adduct peaks b,c,d and f (Fig. 2a) eluted in the same fraction number as did the DMBA–DNA adduct peaks: retention times were 22–27, 28–35, 71.5–74.5 and 98–101 min, respectively. Thus, it is important to note that adduct (VI) as observed in DMBA-treated glands is similar to adduct f obtained from 7-OHM-12-MBA-treated glands in regard to retention time. Although two relatively minor peaks, a and e (Fig. 2a), were absent in the DMBA–DNA adduct profile (Fig. 1a). The major adduct, peak f, contained [mean (S.D)] (28.3 2.2%) of the methanol-soluble material chromatographed in Fig. 2a. The presence of β-carotene (10^{-6} M) inhibited the adduct f by 96.1%. However, influence of β-carotene on the remaining adducts a–e remained unaltered (Fig. 2b). Based upon reverse-phase HPLC data of the fractions obtained from Servacel DHB, adduct (VI) was not retained by the boronate column and this confirms that adduct (VI) is a syn-dihydrodiol epoxide product (data not shown).

Effect of β-carotene on the formation of DMBA metabolites

Figure 3 shows the HPLC elution profile of different DMBA metabolites extracted from the glands exposed to the DMBA carcinogen in organ culture for 24 h both in the absence and in the presence of 1 μmol/l β-carotene. Several metabolites of DMBA were tentatively identified as: DMBA *trans* 3,4-diol, DMBA *trans* 8:9 diol, 7-OHMe-12-MeBA and DMBA phenols. The presence of β-carotene did not change the pattern of different metabolic profiles. The levels of formation of metabolites, 7-OHMe-12-MeBA and DMBA *trans* 3,4-diol were significantly affected by the presence of β-carotene. β-carotene increased the rate of formation of DMBA *trans* 3,4-diol by 3-fold, whereas 7-OHMe-12-MeBA was reduced by 5-fold. However, β-carotene did not show any influence on the other metabolites. This inhibitory effect of β-carotene on the formation of 7-OHMe-12-MeBA most likely reflects the inhibition of a minor metabolic pathway of activation of DMBA through the 7-OHMe-12-MeBA.

β-carotene influence on adducts formation during initiation and postDMBA treatment period

In order to determine the effect of β-carotene on the levels of DMBA–DNA adducts formed during initiation and postDMBA treatment period, mammary glands in organ culture were treated with β-carotene during a defined stage of initiation and also

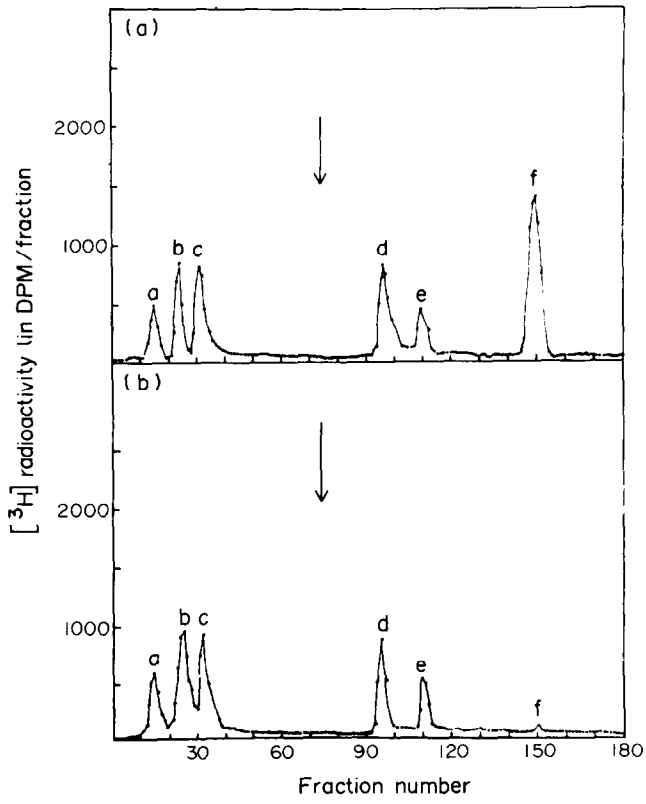

Fig. 2. Effect of β-carotene on the reverse-phase HPLC elution profiles of 7-OHM-12-MBA-induced DNA adducts. Mammary glands in organ culture were exposed to [^{3}H]-7-OHM-12-MBA (11.6 μmol/l 740 KBq/ml) in the absence (a) and in the presence (b) of β-carotene (10^{-6} mol/l). In (a), 25,875 DPM and in (b), 22,215 DPM were injected into the column. The arrow indicates the elution position of toluene used as a positional marker.

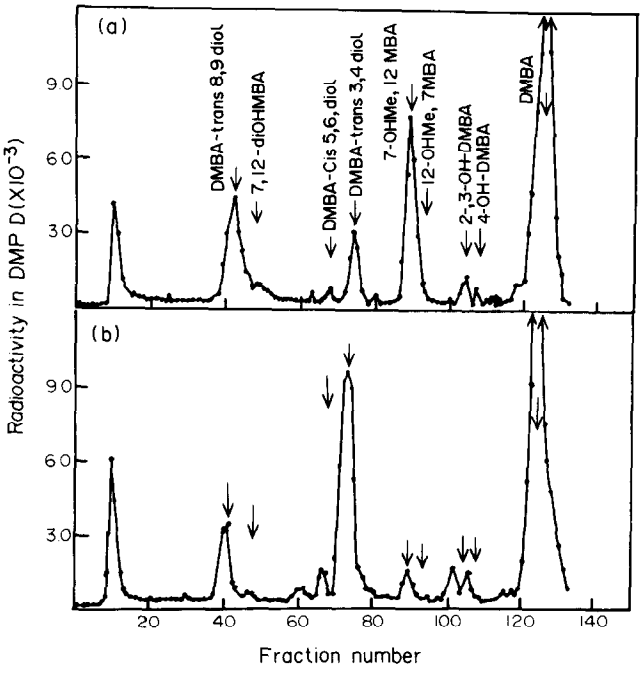

Fig. 3. HPLC profiles of ethylacetate: acetone (2:1) intractable DMBA metabolites obtained after digestion with formic acid (88%) from mammary glands in organ culture treated with *n*-hexane (as control) (a) or with β-carotene (1×10^{-5} mol/l) (b). The column was maintained at ambient temperature with a constant flow rate of 1 μl/min. The gradient system consists of four steps: (i) 50% to 70% methanol-in-water gradient (linear, 20 min), (ii) 10 min hold at the 70% methanol-in-water, (iii) 70–90% methanol-in-water gradient (linear, 20 mins) and (iv) 90–100% methanol-in-water (linear for 3 mins). Individual 0.4 min fractions were collected immediately after injection of samples into the HPLC column. Total dpm injected were between 50 000 and 75 000.

during the postDMBA treatment period (see Materials and Methods). Analysis of DMBA–DNA adducts had demonstrated that β-carotene (10^{-7}–10^{-5} M) caused 85.2–94.8% and 69.5–88.3% inhibition of formation of the adduct (VI) in a dose-dependent manner both at initiation and during the postDMBA treatment period, respectively (Table 1). However, the levels of other adducts I to V which includes the three major characterised adducts, III, IV and V remained constant at both stages. Inhibition of adduct (VI) caused by β-carotene was attributed to the decrease of the ratio of syn- and anti-adducts.

Influence of α-tocopherol and canthaxanthin on DMBA–DNA adducts in mammary glands

In order to further ascertain the mechanism of inhibition of adduct by β-carotene we used α-tocopherol (a known antioxidant) and canthaxanthin (a non-provitamin A carotenoid) to see whether these compounds have any modifying influence on the levels of the adducts. Both α-tocopherol and canthaxanthin were incapable of inhibiting adduct (VI) (data not shown). However, α-tocopherol showed a dose-dependent inhibition of adducts from I to V, but canthaxanthin had no influence on either of these adducts. The ratio of syn- and anti-DMBA-diol-epoxide-deoxyribonucleoside adducts also remained constant both in the absence and in the presence of various concentrations of the compounds tested.

DISCUSSION

Retinoids have been classified as a potential inhibitor because they can inhibit carcinogenesis when administered prior to or after exposure to the carcinogen [19]. In the present study we have analysed the effect of β-carotene on the formation of DNA adducts from [^{3}H]DMBA and [^{3}H]-7-OHM-12-MBA in the mouse mammary gland in organ culture. Results showed that the β-carotene-sensitive adduct derived from [^{3}H]DMBA (Fig. 1a, adduct peak VI) in mammary gland has a retention time identical to that of DNA adduct peak derived from [^{3}H]-7-OHM-12-MBA (Fig. 2a, adduct peak f), indicating adduct (VI) and adduct f are similar. Moreover, it is also important to mention that β-carotene-sensitive adduct (VI) was not present when adducts derived from DMBA *trans* 3,4-diol-treated mammary glands were analysed (results not shown). Immobilised boronate chromatographic analysis of DMBA–DNA adducts further revealed that the β-carotene-sensitive adduct peak is a syn-product. Thus, the evidence suggests that the β-carotene-sensitive syn-adduct is derived from further metabolism of 7-OHM-12-MBA, an intermediate of the DMBA metabolic pathway. There have been several reports that monohydroxymethyl-7,12-dimethylbenz(a)anthracene-DNA adducts formed in mammary gland [20] and liver [21] after administration of DMBA to female Sprague–Dawley rats. DiGiovanni *et al.* also reported the formation of DMBA adducts derived from 7-OHM-12-MBA after treatment of DMBA to Sencar mice, in mouse epidermis [22]. In contrast, no DNA-binding products derived from 7-OHM-12-MBA were detected in mouse skin [23] or mouse embryo cells in culture [24] after administration of DMBA.

Table 1. *Effect of β-carotene on the levels of formation of different adducts during initiation and postDMBA treatment period* in vitro

Treatment of β-carotene (μmol/l)	Initiation period* Adducts (pmol/mg DNA)‡								PostDMBA treatment period† Adducts (pmol/mg DNA)‡							
	I	II	III	IV	V	VI		Syn/anti	I	II	III	IV	V	VI		Syn/anti
0	0.72	0.90	7.55	2.82	4.20	1.35		0.35	0.15	0.24	1.65	0.62	0.92	1.28		0.74
0.1	0.70	0.92	7.53	2.89	4.05	0.20	(85.2)	0.27	0.14	0.25	1.63	0.60	0.90	0.39	(69.5)	0.39
1.0	0.68	0.89	7.60	2.80	4.01	0.09	(93.3)	0.25	0.13	0.22	1.60	0.61	0.89	0.22	(82.8)	0.33
10.0	0.70	0.88	7.47	2.79	4.10	0.07	(94.8)	0.25	0.10	0.25	1.58	0.60	0.90	0.15	(88.3)	0.30

* Glands were incubated with β-carotene and DMBA for 24 h (7.8 μmol/l 740 KBq/ml) between the 3rd and 4th day of culture, then thoroughly washed with DMBA-free medium and incubated in fresh DMBA-free medium with β-carotene for 3 h (total time period 27 h).

† β-carotene was added at the end of initiation period (27 h after the addition of DMBA) in DMBA-free medium and incubated for 24 h.

‡ Values of each adduct were obtained from two or three pooled samples. Numbers in parenthesis indicate the percentage of inhibition.

β-carotene-mediated inhibition of adduct from mammary gland in organ culture is different from an earlier report [25] on selenium-mediated inhibition of adduct in fetal mouse cell cultures. In the latter case, selenium inhibits anti-dihydrodiol epoxide adducts. Syn-dihydrodiol epoxide adducts were not affected. β-carotene-mediated adduct inhibition also seems to be different from the action of known antioxidant, α-T or non-provitamin A carotenoid, CTX. When β-carotene was used for the prevention of DMBA, carcinogenesis inhibited adduct (VI) both at the initiation and during the postDMBA treatment periods (Table 1). This observation is consistent with our earlier studies that show treatment of β-carotene during and after exposure of mammary glands to DMBA caused the inhibition of DMBA-induced transformation *in vitro* [6]. Thus, the inhibition of adducts by β-carotene may allow an insight into how it is acting as a preventive agent in DMBA-mediated initiation of transformation of mammary glands *in vitro*.

Carcinogen-induced DNA adducts are considered to be potent tumour initiators [8, 9]. Thus, the modifying action of β-carotene on the adducts formation is likely to be protective against tumour formation. However, in the present studies, modifying influence of β-carotene on adduct (VI) in relation to tumorigenesis remains unclear at this time.

Although several tissues like lung, liver and skin can metabolise β-carotene to retinol [26], the ability of the mammary cells to support metabolism of β-carotene is unknown. Earlier, we reported that a 10^{-5} mol/l concentration of β-carotene resulted in an undetectable level of retinol accumulation in the treated mammary cells in organ culture [6]. Thus, the mechanism of inhibition by β-carotene is independent of its conversion to retinol in the mammary glands *in vitro*. β-carotene can inhibit peroxidation of unsaturated lipids [2, 27]. Evidence indicates that activation of PAH derivatives to ultimate carcinogenic molecules through epoxidation may occur as a result of microsomal lipid peroxidation [28]. Furthermore, promoting agents can also induce lipid peroxidation [29] and acting through this pathway may eventually promote neoplasia [30]. Lipid peroxidation also can be expected to have severe consequences on the cell such as the release of many biological factors, including prostaglandins. Thus, evidence suggests the action of β-carotene may be mediated through a lipid peroxidation pathway. It is conceivable that a DNA repair mechanism may also play a role in the β-carotene-mediated adduct inhibition.

In conclusion, present results provide the evidence that β-carotene can inhibit an adduct that forms through a 7-OHM-12-MBA intermediate during DMBA carcinogenesis of mammary cells *in vitro*, and the compound acts both at initiation and postDMBA treatment period.

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Establishment and Characterisation of a Human Glioma Cell Line

Claude Chauzy, Bertrand Delpech, Annie Olivier, Christian Bastard, Nicole Girard, Marie-Noëlle Courel, Catherine Maingonnat, Thierry Frébourg, Jean Tayot and Pierre Creissard

A new cell line, CB109, has been established from a human glioblastoma multiforme. The cytoskeleton was positive for glial fibrillary acidic protein, vimentin and fibronectin. Hyaluronan (HA) and the HA-binding protein hyaluronectin (HN) were expressed in the cell cytoplasm and in the extracellular matrix of spheroids and plated cells. Hyaluronidase did not prevent spheroid formation suggesting that HA was not involved in the cell–cell adhesion. HA precoating prevented cell adherence to the plates and favoured spheroid formation. HA was secreted in relatively large amounts into the culture medium. High performance liquid chromatography demonstrated that HA was in the high molecular weight form. The rate of HN secretion by cells was very low. Basic fibroblast growth factor significantly increased the proliferation *in vitro* and tumour growth after grafting into nude mice. The epidermal growth factor receptor was not expressed on cultivated CB109 cells. Cytogenetic analysis showed polysomy 7, structural rearrangement of chromosome 10 short arm and a translocation 13q13-q14 without detectable alteration of the RB gene.
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INTRODUCTION

MALIGNANT GLIOMAS are relatively easy to establish as continuous cell lines [1, 2]. Glioma-derived cell lines offer the opportunity to analyse different aspects of tumour response *in vitro* and *in vivo*. We have cultivated 120 human primary tumours and isolated 10 glioma-derived cell lines. One of them (CB109), initiated from a human glioblastoma multiforme, exhibits several interesting features which are: cytoplasmic markers, hyaluronic acid secretion, basic fibroblast growth factor (bFGF) sensitivity and karyotype pattern. No RB gene alteration was detected.

MATERIALS AND METHODS

Origin of cell line

The CB109 cell line was derived from a left parietal glioblastoma multiforme of a 58-year-old man, taken at surgery.

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Histology

Tissues from the original tumour were fixed in liquid nitrogen. Tissues from the grafted tumours were fixed in acetic alcohol (1% glacial acetic acid in 95° alcohol) or 4% formalin. Sections were stained with haematoxylin and eosin.

Cell culture

A 3 × 2 × 2 cm fragment with a large necrotic and haemorhagic part was obtained from surgery. After removal of the necrotic tissue the remainder was clearly identified as tumour tissue. The material was rinsed twice with Ca^{2+} and Mg^{2+} free Tris buffer, minced with scalpels and allowed to settle. The supernatant was discarded and the pellet disaggregated in culture medium. Cells were separated by gentle aspiration in a 2 ml pipette about 10 times. Following centrifugation at 80 g for 5 min, the pellet was disaggregated in culture medium and the suspension was distributed in four 75 cm^2 tissue flasks (Costar). Primary culture medium was Ham's F10 with 20% heat decomplemented fetal calf serum (FCS), 1% glutamine, 100 U/ml penicillin. The culture was grown in a 5% CO_2 in air incubator, with 100% humidity at 37°C. At the first passage, the FCS concentration in the medium was reduced to 10%. The medium was changed twice weekly until cell confluence, when the cells were removed by a 0.05% trypsin–0.02% EDTA solution in