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journal or publication title	"Heavy oil spilled from Russian tanker ""Nakhodoka"" in 2001 : Towards eco-responsibility, eartb sense"
page range	214-230
year	2003-10-01
URL	http://hdl.handle.net/2297/5535

Endocrine Disrupting Activities of C-Heavy Oil

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ABSTRACT

An oil spill accident happened to a Russian tanker *Nakhodka* with a cargo of heavy oil type C in the Sea of Japan on January, 1997 and the spilled oil severely polluted the coast of Japan and damaged the environment. In this study, androgenic and antiandrogenic activities of C-heavy oil crude extracts prepared with ethanol were evaluated on two androgen-responsive cell lines, a Shionogi mouse mammary carcinoma SC115 and a human prostate carcinoma LNCaP. Oils used in this study were the *Nakhodka* and a commercial C-heavy oils. Assessment of androgenic and antiandrogenic activities was made on the basis of effect on proliferation (SC115) and prostate specific antigen (PSA) production (LNCaP cells) in the absence and the presence of 0.5 nM dihydrotestosterone (DHT). While both extracts exerted almost no effect on the proliferation and PSA production of SC115 and LNCaP cells in the absence of DHT, the extracts significantly inhibited the DHT-induced proliferation and PSA production of the cells in the presence of DHT, indicating that the C-heavy oils contains antiandrogenic compounds. It has been known that androgen receptor (AR) expressed in LNCaP cells has mutation in ligand-binding domain and consequently its transcription promoting action after ligand-binding is different from that of normal AR. Cyproterone acetate, an androgen antagonist, and 17β-estradiol, an estrogen, stimulated PSA production and their stimulatory effects were additive to that of DHT in LNCaP cells, while cyproterone acetate inhibited DHT-induced proliferation and 17β-estradiol showed quite weak agonistic effect in SC115 cells. Therefore, a part of antiandrogenic effects of the oil extracts was considered to be mediated through mechanism other than direct transcriptional activation by activated AR. Then, a few polycyclic aromatic hydrocarbons which are considered not to bind to AR were examined for their androgenic and antiandrogenic effect. Benz[*a*]anthracene, benzo[*k*]fluoranthene and benzo[*a*]pyrene suppressed DHT-induced proliferation and PSA production of SC115 and LNCaP cells in a concentration-dependent manner. The antiandrogenic effect of the two heavy oil extracts was considered to be due in part to polycyclic aromatic hydrocarbons

INTRODUCTION

A Russian tanker *Nakhodka* with a cargo of about 19,000 kl of heavy oil type C met a hull-broken accident in the sea of Japan (about 133°30'E, 37°20'N) on January 2, 1997. While the stern section of the tanker sank down to the sea bottom, the bow section was drifted and grounded on rocks near the Mikuni town, Fukui Prefecture. Amount of the spilled oil was estimated to be more than 6,200 kl (Hatano, 1997). The oil released into the sea was drifted to the coastal regions of eight prefectures from Shimane through Yamagata, Japan. Marine and coastal environment were seriously damaged (EAJ, 1998; Ishikawa Prefecture, 1998; Mizutani, 1997) and long-term toxicological impacts on human and wildlife were important problems, as has been pointed out every time severe oil pollution happened.

An aspect of long-term impacts is a gene toxicity because C-heavy oil contains polycyclic aromatic hydrocarbons (PAHs) at high concentrations (Speight, 1988) and some of them are mutagenic and/or carcinogenic. The authors reported that the crude extracts of the *Nakhodka* heavy oil, spilled oil and commercial C-heavy oil showed mutagenicities in Ames test and the mutagenicities appeared to be due in part to PAHs (Goto *et al.*, 1997; Hayakawa *et al.*, 1997).

Another aspect could be endocrine disrupting action. In recent years, there is increasing concern that man- and nature-made chemicals may cause dysfunction of human and wildlife endocrine systems leading to adverse health effects such as increased rates of specific cancers, reproductive system abnormalities and immune system deficiencies (Cooper and Kavlock, 1997; Kavlock *et al.*, 1996; McLachlan and Korach, 1995; Melnick, 1999). These chemicals have been described as endocrine disruptors. A remarkably wide variety of chemicals including environmental pollutants, industrial chemicals, and natural products are being studied for their effects on endocrine functions, most of these studies have dealt with estrogenic and antiestrogenic activities of substances. On the other hand, the number of environmental chemicals identified with antiandrogenic properties is increasing (Akingbemi and Hardy, 2001). There are also several studies indicating that certain chemicals can impair the function and development of male reproductive systems. Mice exposed *in utero* to hydroxyflutamide, an antiandrogen, showed the inability to impregnate (Silversides *et al.*, 1995). In mice and rats *in utero* and lactational

exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) decreased ventral prostate, seminal vesicle and epididymal weights and daily sperm production (Gray *et al.*, 1995; Hamm *et al.*, 2000; Theobald and Peterson, 1997). Dysfunction of the male reproductive system was found in alligators in Lake Apopka, Fl, USA, which is heavily polluted with DDT and its metabolites (Guillette *et al.*, 1996).

It has been also indicated that some endocrine disrupting effects are not directly mediated through mechanisms that involve nuclear hormone receptors (Ignar-Trowbridge *et al.*, 1995; Harper *et al.*, 1994; Sadar, 1998; Safe *et al.*, 1998). The details of structure-action relationships and molecular mechanisms of endocrine disruptors still remain unclear. C-heavy oil consists of a large number of chemicals and some of constituent PAHs have been listed up as possible endocrine disruptors (Keith, 1998). Although it is probable that C-heavy oil shows endocrine disrupting action and this is an important problem involved in oil pollution, only little knowledge has been obtained yet.

The authors first reported in the preceding paper that crude extracts of the *Nakhodka* and commercial C-heavy oils showed antiestrogenic effect in human breast cancer MCF-7 cells (Kizu *et al.*, 1999). In this study, crude extracts of the C-heavy oils were examined for androgenic and antiandrogenic effect using two androgen-responsive cell lines, a Shionogi mouse mammary carcinoma SC115 and a human prostate carcinoma LNCaP, in order to get further insight on long-term toxicological impacts by C-heavy oil pollution.

METHOD

Chemicals

C-heavy oils examined in this study are an oil drawn from hold of *Nakhodka* after the hull-broken accident and a commercial C-heavy oil which was generously provided from Environmental Protection Center, Kanazawa University. Ethanol of ultra pure grade from Wako Pure Chemicals (Tokyo, Japan) was used throughout. Unless specified otherwise, all other chemicals were of reagent grade or better from commercial sources and used as received. Stock solutions of dihydrotestosterone (DHT), cyproterone acetate (CA) and 17β-estradiol (E2) were prepared at a

concentration of 1 mM with ethanol, being stored under refrigeration and diluted with ethanol to give the desired concentrations prior to use.

Preparation of crude extract of C-heavy oil

Crude extracts of C-heavy oils were prepared as described previously (Kizu *et al.*, 1999). Briefly, 20 ml of ethanol was added to 1 g of oil. The resulting solution was stirred for 30 min at room temperature. Ethanol phase was evaporated to dryness *in vacuo*. The residue was dissolved with ethanol at the extract concentration of 20 mg/ml. The extracts were stored under refrigeration and diluted with ethanol prior to use. The extracts prepared from *Nakhodka* and commercial oils were designated as Ex-N and Ex-C, respectively.

Cell culture

Shionogi mouse mammary carcinoma SC115 cells were obtained through the courtesy of Shionogi Co. (Osaka, Japan) and routinely maintained on collagen type I-coated 21 cm^2 culture dishes (Becton-Dickinson; San Jose, CA, USA) in serum-free medium GIT® (Nihon Pharmaceutical; Tokyo, Japan) supplemented with 1 nM DHT. Human prostate carcinoma LNCaP cells were purchased from Dainippon Pharmaceutical (Tokyo, Japan) and routinely maintained in RPMI-1640 medium (Sigma; St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Intergen; Purchase, NY, USA), 50 mg/l streptomycin and 5,000 units/L penicillin. SC115 and LNCaP cells were grown under standard conditions, at 37°C in a humidified atmosphere of 5% CO_2 - 95% air, and passaged with trypsinization every fourth days.

Assay of androgenic and antiandrogenic activities in SC115 cells

In SC115 cells, proliferative effect was assessed in a manner similar to the E-SCREEN assay (Soto *et al.*, 1995). SC115 cells were plated on collagen type I-coated 24-well culture plates (Becton-Dickinson) at a density of 1 x 10^4 cells per well and cultured for 1 day in GIT® medium not supplemented with DHT. Then, the cells were treated with Ex-N, Ex-C, CA or E2 in the absence or presence of 0.5 nM DHT for 2 days. Cell numbers in wells were measured by a colorimetric MTT method (Mosmann, 1983) and the relative proliferative effect (RPE) was calculated using a following

equation,

$$RPE = 100 \times (Abs_s - Abs_b) / (Abs_{pc} - Abs_b)$$

where Abs_s, Abs_{pc} and Abs_b are absorbances of sample well, positive control well (0.5 nM DHT) and blank well (ethanol only) at 540 nm, respectively.

Assay of androgenic and antiandrogenic activities in LNCaP cells

In LNCaP cells, effects on prostate specific antigen (PSA) production was examined. LNCaP cells were plated on 48-well culture plated at a density of 2×10^4 cells per well and cultured for 1 day in routine culture medium and then 1 day in assay medium composed of phenol red-free RPMI-1640 (Sigma) supplemented with 5% charcoal-stripped FBS, 50 mg/l streptomycin and 5,000 units/l penicillin. Cells were treated with Ex-N, Ex-C, CA or E2 in the absence or presence of 0.5 nM DHT for 5 days. Aliquots of media were withdrawn for analysis of prostate specific antigen (PSA) released into the medium. Cell numbers were measured as described above. PSA concentrations were determined by sandwich-type enzyme immunoassay (Kuriyama et al., 1980) using "E-PLATE EIKEN PSA" kit (Eiken Chemicals; Tokyo, Japan) and normalized to cell number.

Statistical analysis

Statistical analyses were made using unpaired Student's *t*-test with StatView 4.0 for Macintosh computer (Nankodo, Tokyo, Japan), whereby a value $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Effects of the oil extracts on the proliferation of SC115 cells

Shionogi mouse mammary carcinoma SC115 is a novel androgen-responsive cell line (Mineshita *et al.*, 1980; OECD, 1997), while only few cell lines have been known to be androgen-responsive. SC115 cells have been mainly used in studies on action mechanisms or chemotherapeutic efficacies of antitumor agents (Kerr *et al.*, 1999;

Ohigashi *et al.*, 1999) but not in studies on endocrine disruptors. Then, responsiveness of SC115 cells to an androgen (DHT), an androgen antagonist (CA) and an estrogen (E2) were first examined. The responsiveness was evaluated on the basis of effect on proliferation because it has been unknown what androgen-regulated genes are expressed in SC115 cells. When the cells were cultured in the presence of 1 nM DHT, cell number increased by a factor of about 10 for 2 day cultivation and by a factor of about 30 for 3 day cultivation over the initial cell number, the starting cell density and cultivation time were fixed at 1 x 10^4 cells per well and 2 days, respectively, taking into account the growth rate and capacity of a well of 24-well culture plate. Figure 1 shows the cell numbers of SC115 cells cultured at different DHT concentrations. Growth rate of the cells increased with an increase of DHT concentration and reached the maximum

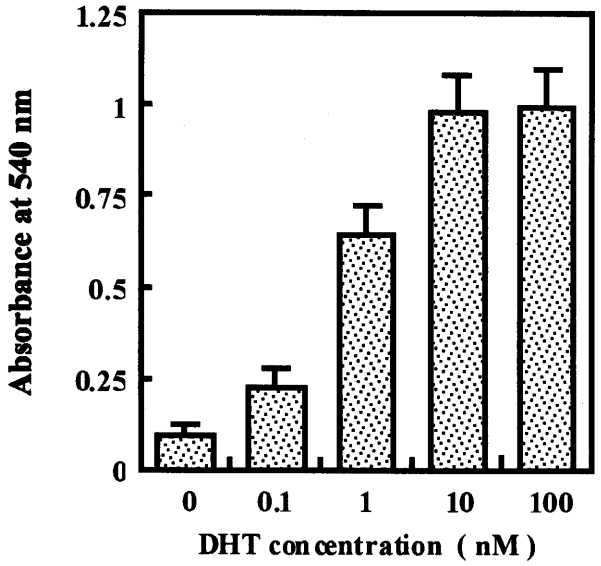

Figure 1 Proliferation of SC115 cells at different DHT concentration. Each column represents the mean and SD, respectively, from three separate cultures.

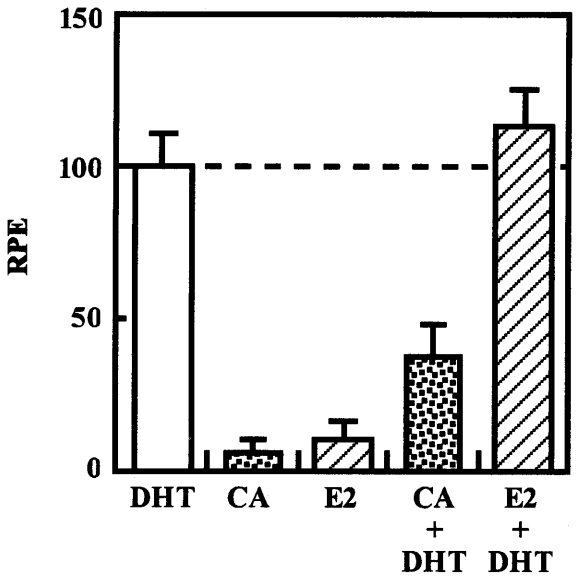

Figure 2 Effects of CA and E2 on PRE of SC115 cells in the absence and presence of DHT. Concentrations: CA, 10 nM; E2, 100 nM; DHT 0.5 nM. Each column represents the mean and SD, respectively, from three separate cultures.
* $p < 0.05$, significantly different from blank.
** $p < 0.05$, significantly different from positive control.

at 10 nM. The proliferation of SC115 cells was confirmed to be dependent on DHT concentration. DHT concentration of the positive control used in calculation of RPE was set at 0.5 nM which was considered to give a growth rate about half the maximum so that agonistic and antagonistic effects of test samples can be observed. Figure 2 gives the effects of CA and E2 on proliferation of SC115 cells in the absence and presence of 0.5 nM DHT. CA antagonized the DHT-induced proliferation in the presence of DHT, showing almost no effect in the absence of DHT. E2 showed agonistic effect, but quite weak, at the E2 concentration of 100 nM in the absence and presence of DHT. These results indicate that SC115 is a suitable cell line for evaluating androgenic and antiandrogenic effects of test samples, although only E2 was used in this study with regard to steroid hormones other than androgen.

Figure 3 represents the effects of Ex-N and Ex-C on proliferation of SC115 cells in the absence and presence of 0.5 nM DHT. While both Ex-N and Ex-C showed no effect in the absence of DHT, they significantly depressed the DHT-stimulated cell growth in the presence of DHT. It was found that Ex-N and Ex-C, as a whole, showed antiandrogenic effect in SC115 cells, in other words, C-heavy oils contain antiandrogenic chemicals.

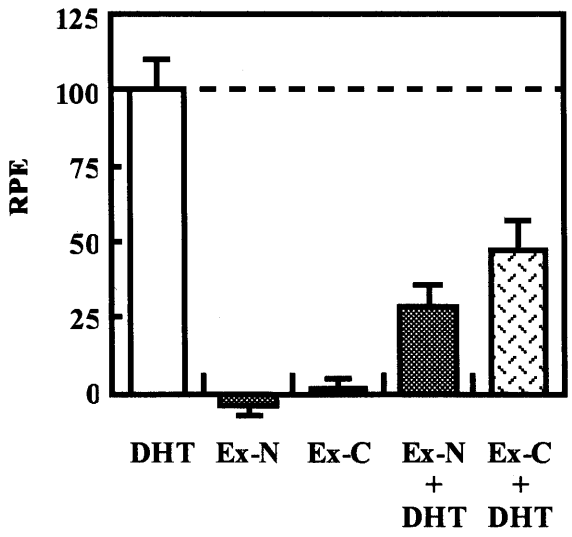

Figure 3 Effects of Ex-N and Ex-C on RPE of SC115 cells in the absence and presence of DHT. Concentrations: Ex-N and Ex-C, 10 μg/ml; DHT, 0.5 nM. Each column represents the mean and SD, respectively, from three separate cultures. * $p < 0.05$, significantly different from positive control.

Effects of the oil extracts on the PSA production of LNCaP cells

Androgen receptor (AR) is a transcriptional factor regulating the expression of

some genes and an androgen is an activating factor of AR (Ing and O'Malley, 1995). Therefore, it is preferable to evaluate the androgenic and antiandrogenic effect by examining expression of androgen-regulated genes. A human prostate carcinoma LNCaP is also androgen-responsive cell line derived from a metastatic lesion of human prostatic adenocarcinoma (Horoszewicz *et al.*, 1980). It has been known that *PSA* gene, an androgen-regulated gene, is retained and expressed in LNCaP cells (Papsidero, *et al.*, 1981). Next, Ex-N and Ex-C were examined for their effects on PSA production of LNCaP cells. PSA is a protein secreted out of cell and accordingly PSA concentration in medium is dependent on cell number in a well. So, PSA concentration was normalized to cell number.

The other hand, AR of LNCaP cells has been known to have mutation in its ligand-binding domain (Thr^{877} to Ala) (Veldscholte *et al.*, 1990). So, the responsiveness (PSA production) of LNCaP cells to DHT, CA and E2 was examined. Figure 4 gives PSA concentrations in wells treated with different concentrations of DHT. As can be seen in Fig. 4, PSA production of LNCaP cells was stimulated by DHT in a concentration-dependent manner. Then, DHT concentration of the positive control was set at 0.5 nM so as to give PSA concentration half the maximum, as in a case of SC115 cells. Figure 5 shows effects of CA and E2 on PSA production in the absence of and presence of 0.5 nM DHT. Both CA and E2 stimulated the PSA production in the absence of DHT and their stimulatory effects were additive to that of DHT. These results indicate that AR of LNCaP cells has quite poor ligand selectivity and antagonists and steroid hormones other than androgen also act as androgen. Similar results were obtained on vinclozolin, a fungicide (Wong *et al.*, 1995). Although it was apparent that LNCaP cell line was

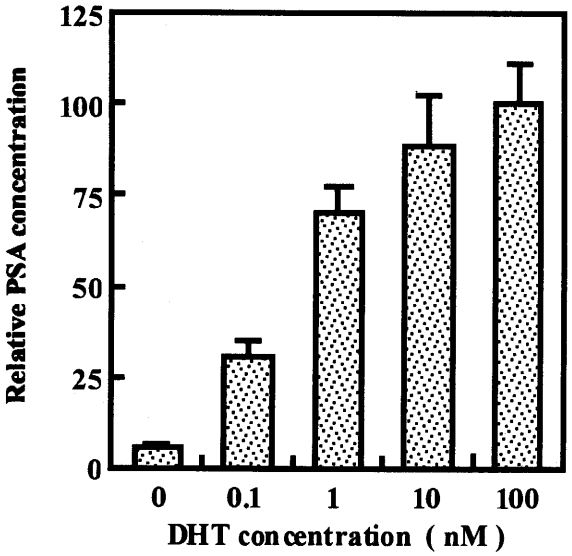

Figure 4 PSA production of LNCaP cells at different DHT concentration. Each column represents the mean and SD, respectively, from three separate culture.

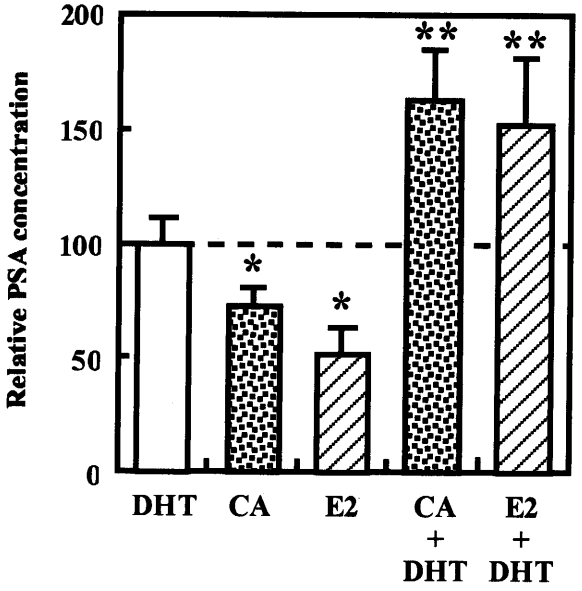

Figure 5 Effects of CA and E2 on PSA production of LNCaP cells in the absence and presence of DHT. Concentrations: CA, 10 nM; E2, 100 nM; DHT 0.5 nM.
Each column represents the mean and SD, respectively, from three separate cultures.
* $p < 0.01$, significantly different from blank.
** $p < 0.05$, significantly different from positive control.

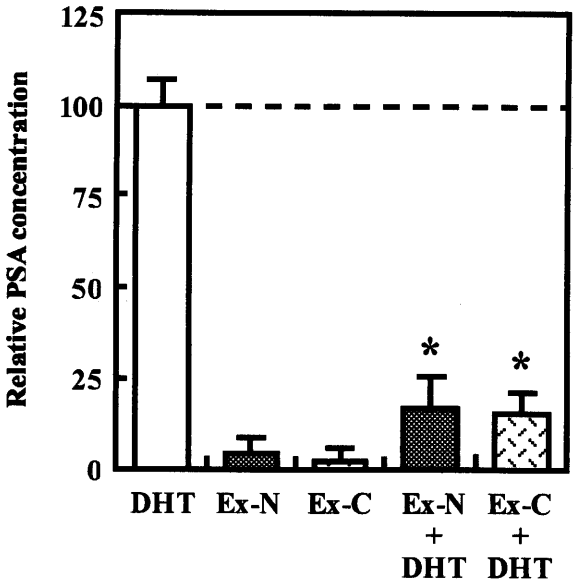

Figure 6 Effects of Ex-N and Ex-C on PSA production of LNCaP cells in the absence and presence of DHT. Concentrations: Ex-N and Ex-C, 10 μg/ml; DHT, 0.5 nM.
Each column represents the mean and SD, respectively, from three separate cultures.
* $p < 0.01$, significantly different from positive control.

unsuitable for studies on endocrine disruption mediated by AR, the authors examined the effect of Ex-N and Ex-C on LNCaP cells. Figure 6 represents the effect of Ex-N and Ex-C on PSA production in the absence and presence of DHT. As is distinct from the effect of CA and E2, Ex-N and Ex-C did not induce the PSA production in the absence of DHT, moreover, they significantly depressed the DHT-induced PSA production in the presence of DHT. The oil extracts were found to show antiandrogenic effect even on LNCaP cells. If the action of AR activated by oil constituents is dominant in the cells, PSA production should be stimulated as seen on CA and E2. The results above suggest that a part of the antiandrogenic effect of Ex-N

and Ex-C is mediated by mechanism other than direct transcriptional activation by activated AR

Effects of BaA, BkF and BaP on proliferation of SC115 cells and PSA production of LNCaP cells

As mentioned above, some endocrine disrupting mechanisms in which nuclear hormone receptors are not involved have been investigated (Ignar-Trowbridge *et al.*, 1995; Harper *et al.*, 1994; Sadar, 1998; Safe *et al.*, 1998). The authors have reported that Ex-N and Ex-C contain a number of PAHs at high concentrations (Hayakawa *et al.*, 1997) and show antiestrogenicity in MCF-7 cells (Kizu *et al.*, 1999). Some PAHs

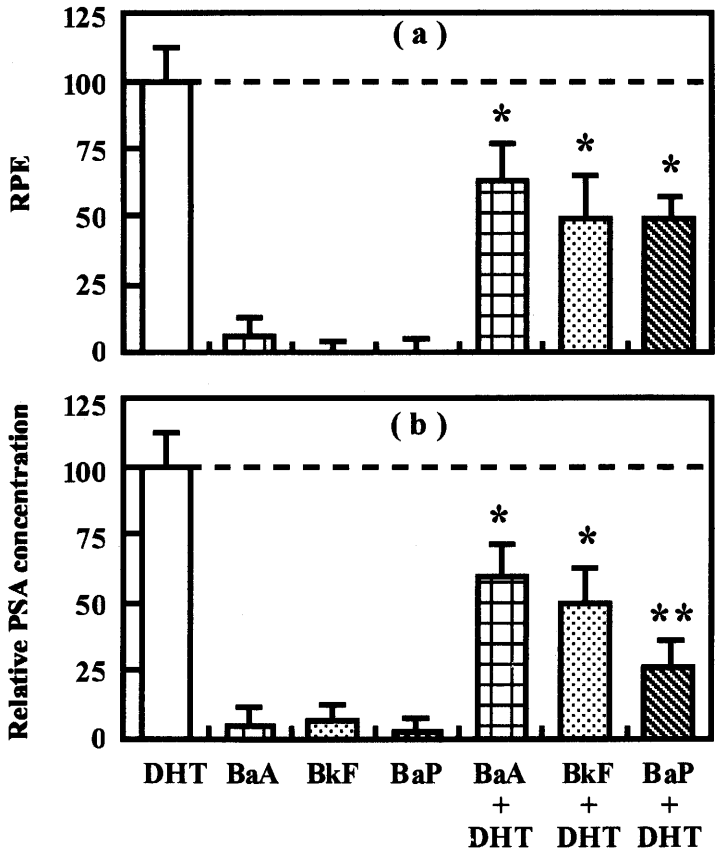

Figure 7 Effects of BaA, BkF and BaP on the proliferation of SC115 cells (a) and PSA production of LNCaP cells (b) in the absence and presence of DHT
Concentrations: BaA & BkF & BaP, 1 μM; DHT 0.5 nM. Each column represents the mean and SD, respectively, from three separate cultures.
* $p < 0.01$, significantly different from positive control.
** $p < 0.01$, significantly different from positive control.

including benzo[*a*]anthracene (BaA), benzo[*k*]fluoranthene (BkF) and benzo[*a*]pyrene (BaP) have been listed as possible endocrine disruptors (Keith, 1998).

Then, BaA, BkF and BaP were examined for their androgenic and antiandrogenic effect on SC115 and LNCaP cells. Shown in Fig. 7 are the effects of BaA, BkF and BaP on proliferation of SC115 cells (a) and PSA production of LNCaP cells in the absence and presence of DHT. While the PAHs showed no effect on both SC115 and LNCaP cells in the absence of DHT, they significantly suppressed the DHT-induced proliferation and PSA production in the presence of DHT. The suppression got stronger with increasing PAH concentration with each compound (data are not shown). These results indicate that PAHs in the extracts are responsible, at least in part, for the antiandrogenic effects of Ex-N and Ex-C.

Vinggaard *et al.* (2000) also found that certain PAHs elicited antiandrogenic effects in LNCaP and CHO-K1 cells, respectively. PAHs did appear to exhibit the antiandrogenic effects through the activation of aryl hydrocarbon receptor (AhR). Previous works documented that TCDD induces expression of c-fos and c-jun genes and consequently increase in a transcription factor activator protein-1 (AP-1) (Hoffer *et al.*, 1996; Puga *et al.*, 1992). On the other hand, Sato et al. have reported that androgenic induction of PSA gene is repressed by elevating AP-1 level with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or c-jun expression vector in LNCaP cells and that this effect of AP-1 is based on the inhibition of AR-ARE complex formation by protein-protein interaction between the AR and AP-1 (Sato *et al.*, 1997). Elevated mRNA levels of c-jun and c-fos in LNCaP cells treated with Chr, BkF or BaP were observed in the present study, too. The findings in the present study together with the results documented previously present a possible mechanism for the antiandrogenic effects of Chr, BkF and BaP in LNCaP cells that binding of AR to ARE is inhibited through interaction between AR and AP-1 induced by AhR activated with Chr, BkF or BaP.

Another possible mechanism for the cross-talk between AR and AP-1 has been proposed. It is that CREB (cAMP response element binding protein) binding protein (CBP) functions as a coactivator for AR and that the transcriptional interference between AR and AP-1 is the result of competition for limiting amounts of CBP in

LNCaP cells (Frønsdal *et al.*, 1998). This mechanism could contribute to the antiandrogenic effect of Chr, BkF and BaP observed in the present study.

Adding to the mechanisms described above, interaction between AR and AP-1 may involve a number of mechanisms such as overlapping of DNA binding sites of AR with AP-1 and a composite DNA binding site to which both AR and AP-1 bind. These mechanisms may function in gene-specific or cell-specific manners. To date very little is known about the interaction between AR and AP-1. Further investigation is necessary to understand the potential role of AR-AP-1 interaction in the antiandrogenic effects of PAHs and TCDD.

CONCLUSION

Crude extracts of *Nakhodka* and commercial heavy oils, as a whole, showed antiandrogenic effects on cultured mouse mammary carcinoma SC115 and human prostate carcinoma LNCaP cells. The antiandrogenic effects were considered to be due in part to PAHs involved in the oils. Though only two C-heavy oils were examined in the present study, the antiandrogenic effect would be a common problem of C-heavy and other oils. It has been well known that PAHs with 4 or more rings are chemically and biologically stable. The authors reported that concentrations of several PAHs in sand at an area heavily polluted with Nakhodka oil decreased to only half of those immediately after the accident even 6 months later (Hayakawa *et al.*, 1997). After this, studies at a viewpoint of endocrine disrupting action will be required to get knowledge on long-term toxicological impacts by C-heavy oil pollution.

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (R. Kizu) and a Grant-in-Aid for Scientific Research (B) (K. Hayakawa) from the Ministry of Education, Science, Sports and Culture of Japan. The authors would like to thank Drs. T. Hase and H. Kakishima, Eiken Chemical Co. LTD., for their collaboration in PSA analysis and Shionogi Co. LTD. for kind gift of mouse mammary SC115 cells.

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