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Effect of Chemical Carcinogens on the Replication, Cytolyticity, DNA Synthesis, and Protein Expression of Herpes Simplex Virus in Viral Infected Cells

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ABSTRACT

We investigated effects of several chemical carcinogens, i.e., benzo(*a*)pyrene (BP), 7,12-dimethylbenz (a)anthracene (DMBA), nitrosomethyl urea (NMU), and nicotine on the replication, cytolyticity, DNA synthesis, and protein synthesis of type 1 herpes simplex virus (HSV-1) in viral infected Vero cell monolayers. We observed that the BP and DMBA did not show such activity. All chemical carcinogens did not inhibit the synthesis of viral DNA, but the expression of gamma viral proteins that are expressed from the newly synthesized progeny viral DNA was somewhat notably inhibited by BP and DMBA. However, the synthesis of alpha and beta viral proteins was not altered by the chemical carcinogens. These data indicate that the gamma viral proteins expressed from the newly synthesized DNA in the presence of chemical carcinogens in the culture medium may be defective. This is further supported by the fact that the virus fail to replicate in the presence of these chemical carcinogens, in spite of viral DNA and proteins are somewhat normally synthesized.

Key Words: Herpes simplex Virus, Cancer Tabacco, Chemical carcinogens, Gene expression, DNA

INTRODUCTION

Herpes simplex virus (HSV) has been increasingly incriminated in different forms of cancer in recent years. Type 2 herpes simple virus (HSV-2) is associated with uterine cervix cancer in women. Serologic and epidemiologic studies indicate that HSV-2 specific serum antibody titer is higher in uterine cancer patients than non-cancer individuals who has HSV-2 infection (Nahmias *et al.*, 1970 and Rawls *et al.*, 1976).

Certain human cervical cancer cells contain HSV-2 DNA and RNA, and express HSV-2 antigens (McDougall *et al.*, 1980; Kaufman *et al.*, 1981; and Eglin *et al.*, 1983). Type 1 HSV (HSV-1) is mainly linked to human orofacial squamous cell carcinomas (SCC).

Orofacial SCC patients have higher circulating HSV-1 antibody titer than normal individuals (Shillitoe *et al.*, 1982). HSV-1 RNA was been detected in some oral squamous cell carcinoma specimens (Eglin *et al.*, 1983). The HSV's role in carcinogenesis is further evidenced by its in vitro cell transforming capacity and in vivo tumor producing effect. Malignant transformation of mammalian cells can be experimentally induced by ultraviolet (UV)-irradiated HSV (Duff and Rapp, 1971), fragments of HSV DNA (Jariwalla *et al.*, 1980), and photodynamically inactivated HSV (Li *et al.*, 1975). In fact, the transforming region of HSV-1 (minimum transforming region 1, mtr-1) and HSV-1 (mtr-2 and mtr-3) were found to be located in the left third of the HSV-1 genome and close to the center of the HSV-2 genome (Steele and Shillitoe, 1991). Furthermor, in vivo tumorigenicity and cocarcinogenicity of has been reported (Wentz *et al.*, 1981 and Park *et al.*, 1985, 1986, and 1988). However, HSV must be inactivated to induce oncogenic activity, otherwise the virus can only induce rapid cell lysis before it

produces cell transformation (Duff and Rapp, 1971 and Wentz *et al.*, 1981).

Heavy cigarette smoking and use of smokeless tobacco are associated with high incidence of oral carcinogenesis in human (US DHHS, 1982). Mortality rate form cancer of the oral cavity, pharynx, extrinsic larynx and esophagus in cigarette smokers is significantly higher than the rate of nonsmokers (US DHHS, 1986). The Unites State Surgeon General reported that the chronic use of smokeless tobacco would increase the incidence of oral cancer development (US DHHS, 1986). However, no studies have demonstrated the development of oral cancers by intraoral topical application of smoked tobacco tar or smoked tobacco tar or smokeless tobacco in laboratory animals (Shklar *et al.*, 1985; Park *et al.*, 1986). Therfore, possible involvement of other contributing factors has been suspected in tobacco-related oral malignancies. Among those facotrs, HSV has been given much attention because of its ubiquity and presence in the oral cavity. Up to 95% of population acquire HSV-1 antibodies by age of 10. More than 1/3 of world population suffers form recurrent orofacial herpetic infections. Furthermore, many people excrete infectious HSV virions into the oral cavity during prodromal and overt period of recurrent orofacial HSV infections (Spruance *et al.*, 1984). Therefore, there is an ample opportunity for HSV and water-soluble component of smoked tobacco tar or smokeless tobacco in cigarette smokes or smokeless tobacco users, respectively. More recently, we have found that smoked tobacco tar and water soluble extract of smoked tobacco tar inactivate HSV, and by doing so increase the oncogenic activity of the virus (Park *et al.*, 1986). In the present study we have examined the effect of tobacco-related chemical carcinogens such as 7, 12-dimethylbenz (*a*)anthracene (DMBA), benzo(*a*)pyrene (B (*a*)P), nicotine, and nitrosomethyl urea (NMU) on the replication, DNA synthesis and gene expression of HSV-1.

MATERIALS AND METHODS

Viruses and cell

HSV-1 (F-strain) was obtained form the American Type Culture Collection (ATCC), Rockville, Maryland. The viruses were propagated in Vero cell monolyers and the viral titers were adjusted to 2×10^8 plaque-forming units per milliter (PFU/ml). The stock virus was stored at −80℃ until used. Vero cells purchased form the ATCC were grown in Eagle's minimun essential medium (E-MEM) supplemented with 5% fetal bovine serum (FBS). The monolayers were cultured at 37℃ in a 5% carbon-dioxide atmosphere.

Chemical carcinogens

Chemical carcinogens, i.e., benzo(a)pyrene (BP), nitrosomethylurea (NMU), nicotine, and 7, 12-dimethylbenz(*a*)anthracene (DMBA) were purchased from the Sigma Chemical Company (St. Louis, Missouri). The chemicals were dissolved in dimethyl sulphoxide (DMSO) to prepare stock solutions, and the stock solution was further diluted with culture medium. The concentration of DMSO in the culture medium containing the chemical carcinogens was 0.05%.

Determination of the effect of chemical carcinogens on the replication of HSV

To determine the effect of DMBA, BP, nicotine and NMU on the replication of HSV-1, we inoculated Vero cell monolayser with HSV-1 at multiplicity of infection (m.o.i.) of 5 (5 PFU/cell). One hour after the viral inoculation, the cells were washed with phosphate buffered saline, and culture media containing 0, 10, 25, 50, or 100 μM of DMBA, BP, nicotine, or NMU were added to the cells. The cultures were incubated at 37℃ in a CO_2 incubator for 24 hours. They were frozen and thawed three times in phosphate buffered saline (PBS), collected, and centrifuged at 450g for 5 min. The viral titers were then determined from the supernatant using a plaque assay technique.

Determination of the effects of chemical carcinogens on the cytolyticity of HSV-1

To determine the effect of the chemical carcinogens on the cytolytic activity of HSV-1 in cell-free condition, 1.0 ml of HSV-1 with a titer of 2.0×10^7 PFU/ml was mixed with 1.0 ml of either plain E-MEM (control), E-MEM containing 0.05% DMSO (another control group because culture medium containing chemical carcinogens also has 0.0%

DMSO), or E-MEM containing the chemical carcinogens (10, 25, 50 or 100 μM). The mixtures were than incubated at 37℃ for 0, 1 2, or 6 hours. At the end of the incubation period, the mixtures were ultracentrifuged using a Beckman SW 27 rotor at 55,000 g for 2 hours at 4℃ to precipitate viral particles. Supernatants were removed and the viral pellets were resuspended in PBS; viral titers were determined from the resuspended pellets using the plaque assay technique.

Isolation and analysis of viral DNA

To investigate whether the chemical carcinogen-induced HSV-1 growth inhibition is due to the inhibition of HSV-1 DNA synthesis, we observed the effect of the chemical carcinogens on the synthesis of viral DNA in HSV-1 infected cells. Ninety percent confluent Vero cell monolayers in 60-mm Petri dish were inoculated with HSV-1 at the m.o.i. of 5 (5 PFU/cell) and incubated for 1 hour. The cells were washed with PBS twice, and medium containing 100 μM of DMBA, BP, nicotine or NMU was then added to the cultures along with 1.0 μCi/mi of [^{3}H]-thymidine per dish. After incubation at 37℃ for 24 hours the cells were digested with proteinase K and sodium dodecyl sulfate. The cell lysates were then subjected to Nal isopycnic density gradient ultracentrifugation (Chun and Park, 1987). The tubes were then exposed to ultraviolet light and Polaroid pictures of the viral and cellular bands were taken.

Labeling of proteins and polycarylamide gel electrophoresis

HSV must be inactivated to elicit its oncogenic capacity. However, the inactivated virus must produce certain viral proteins for cell transformation. To study whether chemical carcinogen-treated HSV-1 can express viral protein, following experiments were executed. Vero cells were inoculated with HSV-1 at m.o.i. of 20 (20 PFU/cell) and incubated at 37℃ for 1 hours. The cells were washed and fed with medium containing DMBA, B(*a*)P, nicotine or NMU (50 or 100 μM). The cells were incubated for additional 0.5, 4, 7, or 11 hours and labelled with [^{35}S]-methionine (20 μCi/min; 900 mCi/mM, New England Nuclear, Boston, MA) for 1 hours. The cells were then lysed in sample buffer, and the lysates were subjected to polyacrylamide gel elctrophoresis (PAGE) (Morse *et al.*, 1978). The electrophoretic, staining and autoradiographic techniques used are described elsewhere (Gibson and Roizman, 1974; Spear and Roizman, 1972). The stacker and separation gels contained 3% and 9.25% acrylamide, respectively.

After the completion of the electrophoresis, the gels were fixed, stained with Coomassie blue, dried and then autoradiographed on SB-5 Kodak X-ray film (Eastman Kodak Co., Rochester, NY).

RESULTS

Effect of chemical carcinogens on the replication of HSV

All tested chemical carcinogens inhibited the replication of HSV-1 in a dose-dependent manner: the higher the concentration of chemical carcinogens, the greater the inhibition. Nicotine showed the least anti-herpetic activity, while DMBA showed the most potent effect in this experiment. With 100 μM of DMBA, BP, NMU, and nicotine in the culture medium, the viral yield was less than 5%, 15%, 30%, and 35% of that control group, respectively (Fig. 1).

Effect of chemical carcinogens on the cytolytic activity of HSV

Incubation of HSV-1 at 37℃ for 1, 2 or 6 hours

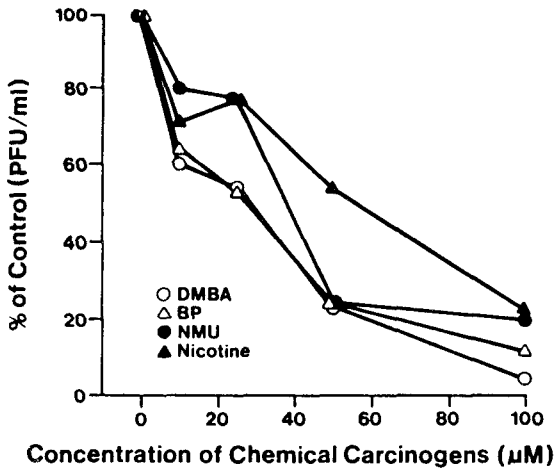

Fig. 1. Effect of chemical carcinogens on the replication of HSV-1 in Vero cell monolayers.

induced an inactivation of the virus, resulting in a significant reduction of its cytolytic activity. In control group (chemical-free control and DMSO groups), temperature inactivation of HSV-1 was obvious at 1, 2, and 6 hours postculture: the cytolyticity of HSV-1 was reduced by 30, 40 and 50%, respectively. In the pressence of DMBA in culture medium, the cytolyticity of HSV-1 was significantly reduced beyond the temperature inactivation in a time-dependent manner: the longer the incubation, the greater inhibiton of cytolyticity. The inhibition was also dose-dependent fashion: The higher concentration of DMBA, the greater inactivation of the virus. In the presence of 100 μM of DMBA, the viral cytolyticity was reduced to 0.7~0.9% of its original activity (Fig. 2). Like DMBA, BP also showed a notable inhibition of HSVA-1 cytolyticity: In the presence of 25-100 μM of BP, the cytolytic activity of HSV-1 was reduced to 2% of its original cytolyticity (Fig. 3). NMU and nicotine also reduced the cellular killing activity of HSV-1, but to efficacy of these two chemicals was much weaker than DMBA or BP: In the presence of 100 μM of NMU or nicotine in culture medium for 6 hours, the cytolytic activity of HSV-1 was reduced to 10% of its original activity (Fig. 4 and 5).

Effect of chemical carcinogens of HSV DNA synthesis

In as much as the viral replication is preceded by viral DNA synthesis, we determined the effect of the chemical carcinogens on the synthesis of viral DNA to find whether the chemical carcinogen-induced inhibition of viral replication is due to the suppression of viral DNA synthesis. Observation of the thickness of viral and cellular DNA bands in ultraviolet light showed that the amound of newly synthesized viral DNA was somewhat decreased in the presence of the chemical carcinogens. However, a significant amount of viral DNA was made in the presence of the chemical carcinogens in the culture medium (Fig. 6). These data indicate that the inhibition of HSV-1 replication by chemical carcinogens may not be associate with the inhibition of viral DNA synthesis.

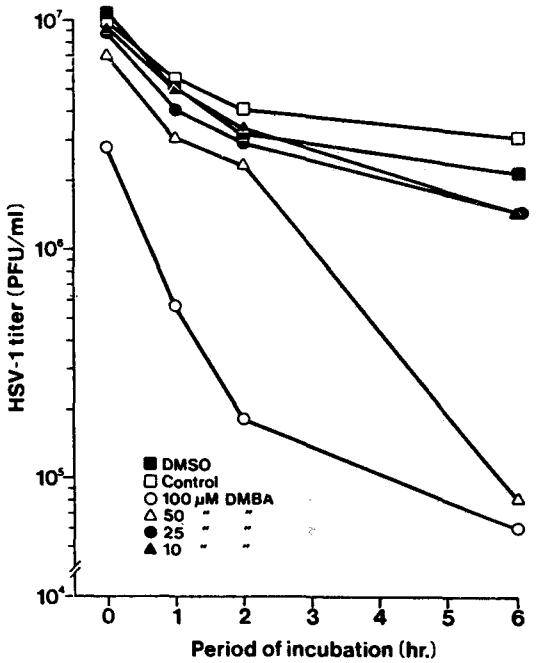

Fig. 2. Effect of 7,12-dimethylbenz(a)anthracene (DMBA) on the cytolytic activity of HSV-1 in cell-free condition.

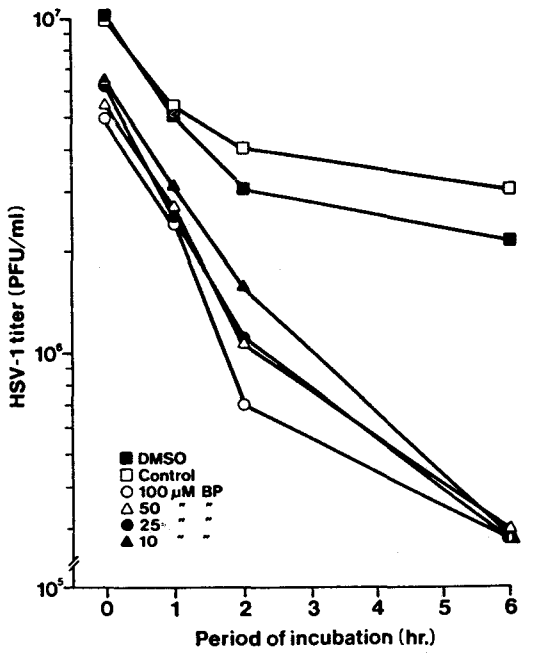

Fig. 3. Effecto of benzo(a)pyrene (BP) on the cytolyticity of HSV-1 in cell-free condition.

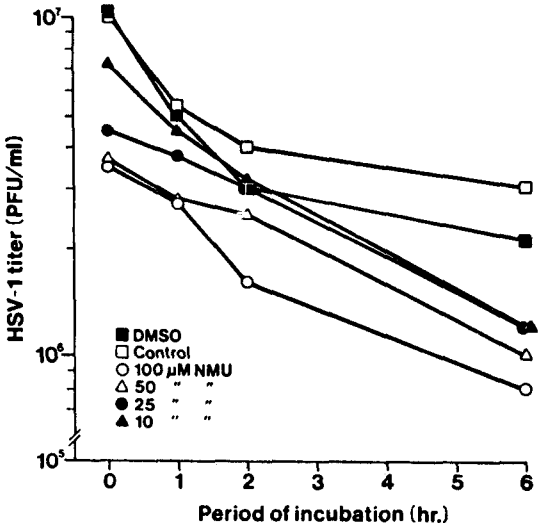

Fig. 4. Effect of nitrosomethyl urea (NMU).

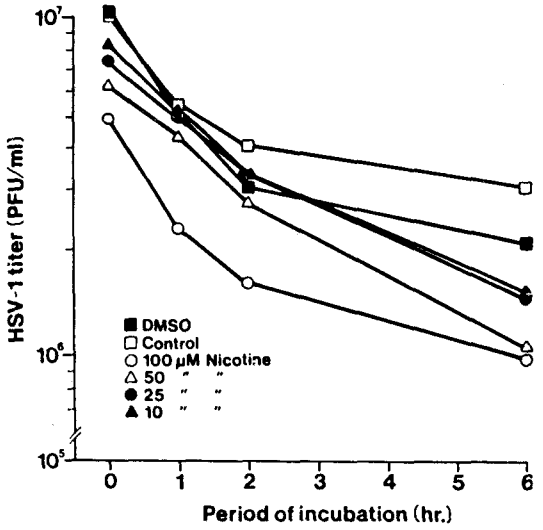

Fig. 5. Effect of nicotine on the cytolyticity of HSV-1 in cell-free condition.

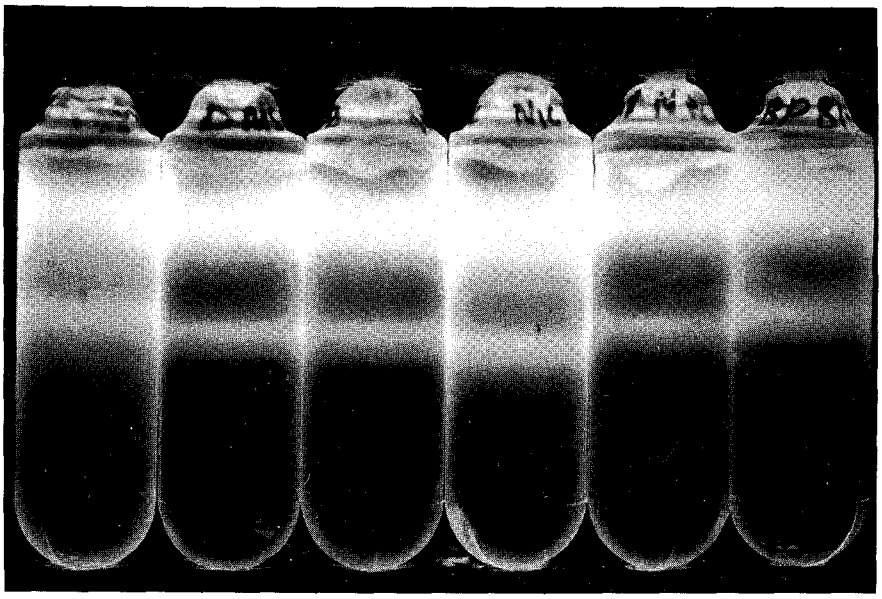

Fig. 6. Effect of chemical carcinogens on the synthesis of HSV-1 DNA synthesis in Vero cell monolayer culture. From left, the ultracentrifugation tubes contain cellular (upper bands) and viral DNA (lower bands) that were synthesized in the presence of control (chemical-free), DMSO, BP nicotine, NMU, and DMBA in the culture medium.

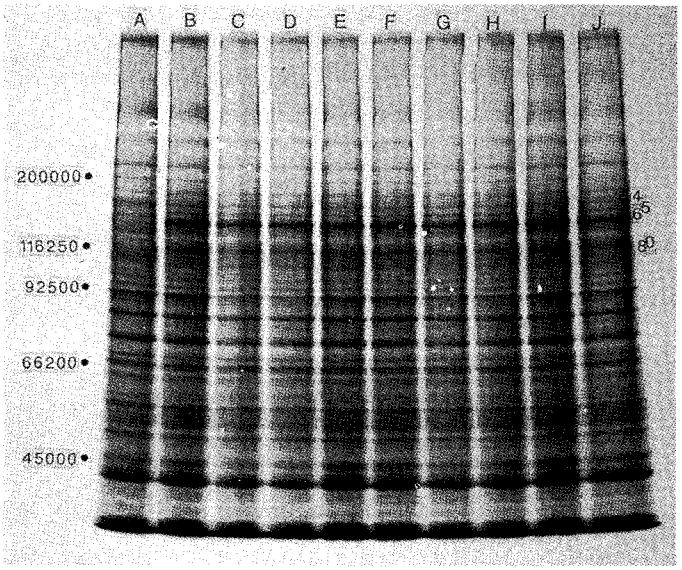

Fig. 7. Autoradiographic images of electrophoretically separated proteins from cells infected with HSV-1 and maintained for 1.5 hrs in normal culture medium (control; lane B) or presence of BP (50 μM, lane C; 100 μM lane D), nicotine (50 μM, lane E; 100 μM, lane F), DMBA (50 μM, lane G; 100 μM, lane H), or NMU (50 μM, lane I; 100 μM, lane J). The lane A is the protein profile of uninfected cells.

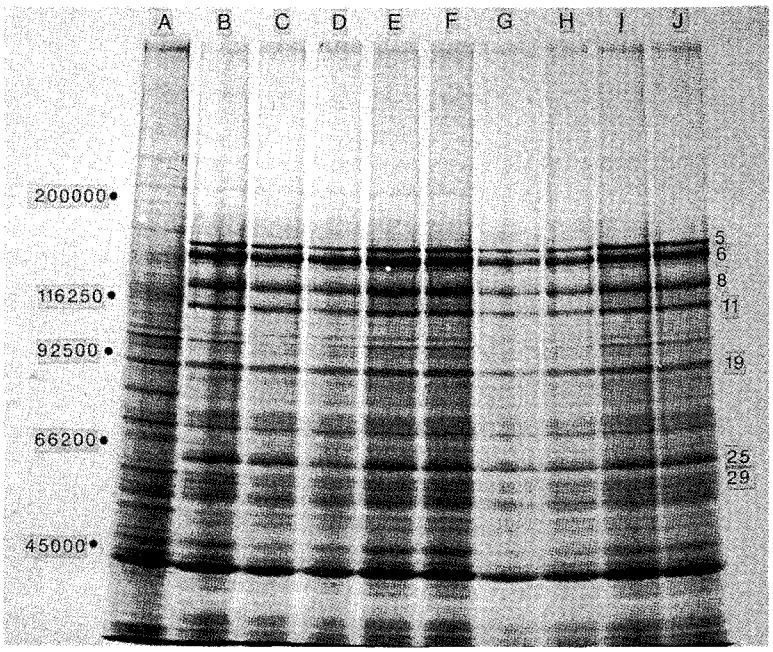

Fig. 8. Autoradiographic images of electrophoretically separated proteins from cells infected with HSV-1 and maintained for 4 hrs in normal culture medium (control; lane B) or presence of BP (50 μM, lane C; 100 μM lane D) nicotine (50 μM, lane E; 100, lane F), DMBA (50 μM, lane G; 100 μM, lane H), or NMU (50 μM, lane I; 100 μM lane J). The lane A is the protein profile of uninfected cells.

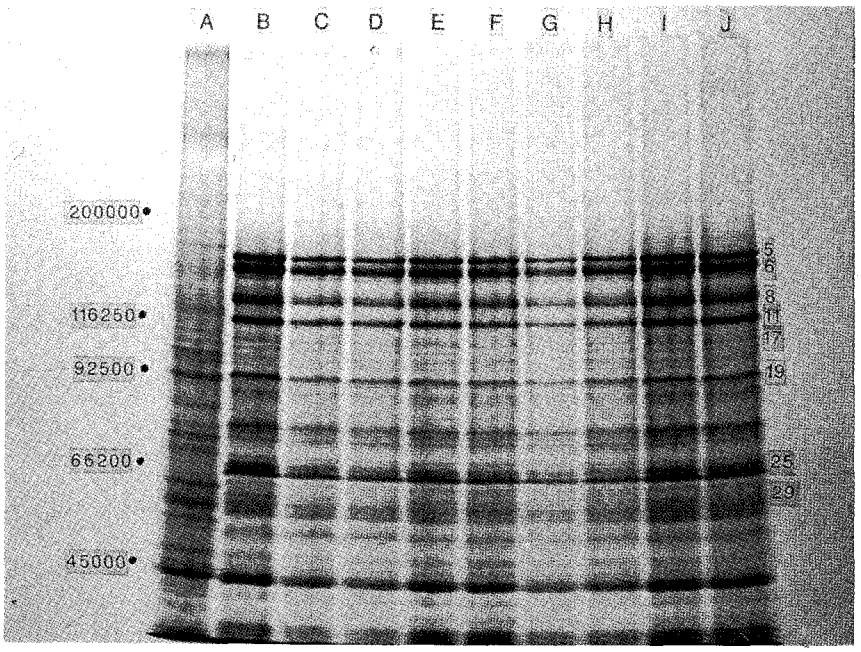

Fig. 9. Autoradiographic images of electroporetically separated proteins from cells infected with HSV-1 and maintained for 7 hrs in normal culture medium (control; lane B) or presence of BP (50 μM, lane C; 100 μM lane D), nicotine (50 μM, lane E; 100 μM, lane F), DMBA (50 μM, lane G; 100 μM, lane H), or NMU (50 μM, lane a; 100 μM, lane J). The lane A is the protien profile of uninfected cells.

However, it is possible that defective viral DNA might be synthesized in the presence of chemical carcinogens, resulting in suppression of replication of the virus.

Effect of chemical carcinogens on the synthesis of HSV-1 protein

The effect of chemical carcinogens on viral protein synthesis is shown with an autoradiogram of polyacrylamide gel slabs containing electrophoretically separated HSV-1 protein (Fig. 7-1). Alpha class protein of HSV (ICP 0) appeared early at 1.5 hrs after the viral infection. Beta class proteins (ICPs 6 and 8) were most abundant at 4 hrs post infection, while gamma proteins (ICPs 5, 11, 17, 19, 15 and 29) appeared most at 7-11 hrs postinfection. Figure 7 shows autoradiographic images of electrophoretically separated proteins from cells infected with HSV-1 and mainatined for 1.5 hours in the absence (control; lane B) or presence of BP (50 μM, lane C; 100 μM lane D), nicotine (50 μM, lane E; 100 μM, lane F), DMBA (50 μM, lane G: 100 μM, H), or NMU (50 μM, lane 1; 100 μM, lane J). The synthesis of infected cell polypeptide 4 (ICP4), ICP5, ICP6, ICP0 and ICP8 was not altered by nicotine and NMU, while DMBA and BP slightly diminished the synthesis of those proteins.

When examined after 4 hrs (Fig. 8), 7 hrs (Fig. 9), 11 hrs (Fig. 10) culture, the synthesis of most viral proteins was significantly diminished by BP and DMBA, but nicotine and NMU did not change the viral synthesis.

DISCUSSION

We previously reported that the water extractable component of smokeless tobacco (snuff-extract) inactivates HSV, and by doing so may increase the viral oncogenic capacity (Stich *et al.*, 1987). Similar to smokeless tobacco, smoked tobacco tar extract inhibited the replication of

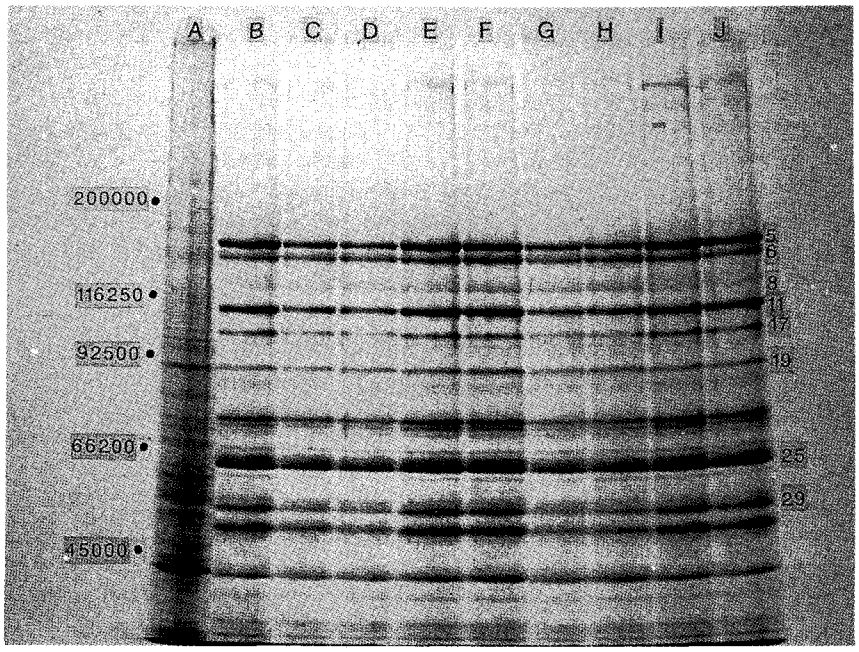

Fig. 10. Autoradiographic images of electrophoretically separated proteins from cells infected with HSV-1 and maintained for 11 hrs in normal culture medium (control; lane B) or presence of BP (50 μM, lane C; 100 μM lane D), nicotine (50 μM, lane E: 100 μM, lane F), DMBA (50 μM, lane G; 100 μM, lane H), or NMU (50 μM, lane I; 100 μM, lane J). The lane A is the protein profile of uninfected cells.

HSV-1 and 2 in Vero cell monolayers. Although at high concentrations of tar extract markedly impaired the cellular metabolism, tar extract, in concentrations which did not interfere with the synthesis of cellular protein and DNA, selectively inhibited viral replication. High concentrated tar extract also inactivated HSV in the cell-free condition and reduced its cytolytic activity.

To investigate whether the tobacco-induced inhibition of viral replication is due to the action of tobacco-related chemical carcinogens, we studied the effect of several chemical carcinogens on the replication, cytolyticity, DNA synthesis and protein synthesis of HSV-1 in viral infected Vero cell monolayers. Similar to snuff-extract and smoked tobacco tar (Oh *et al.*, 1989 and 1990), the chemical carcinogens notably inhibited the replication and cytolyticity of the virus, indicating that the tobacco-induced inactivation of HSV-1 might be, in part, due to the anti-herpetic effect of tobacco-related chemical carcinogens. In as much as the viral replication is preceded by viral DNA synthesis, it is natural to assume that the viral DNA synthesis might be also suppressed by the chemical carcinogens. However, our data show that the viral DNA synthesis was not significantly altered by any of tested chemical carcingens. These results suggest that the inhibiton of viral replication by chemical carcinogens may not be contributed their inhibitory action on viral DNA synthesis. Though the DNA synthesis is not diminished by chemicals, we cannot rule out the possible generation of defective viral DNA by the chemical carcinogen treatment. Because of DNA alkylating activity of these chemical carcinogens, newly synthesized viral DNA might be defective, which results in a production of false viral production. HSV genome expresses nearly 50 viral proteins.

These proteins are classified into 3 groups as alpha, beta, and gamma proteins, whose syntheses is coordinately regulated and sequentially ordered in a cascade (Honess and Roizman, 1975). In the absence of prior viral protein synthesis the alpha proteins are synthesized during the infec-

tious cycle and play a role in the regulation of subsequent beta proteins. Beta proteins, whose synthesis is successed by viral DNA replication, include enzymes necessary for viral DNA synthesis such as thymidine kinase and DNA polymerase (Honess and Roizman, 1975). Our data show that the synthesis of alpha and beta viral proteins was not altered by chemical carcinogens, while the production of gamma viral proteins were significantly inhibited by BP and DMBA, two chemical carcinogens which significantly decreased the replication HSV-1. In as much as the viral DNA synthesis is not altered by these two chemical carcinogens, the newly synthesized viral DNA that is synthesized in the presence of BP and DMBA may be defective.

Repeated exposure of oral mucosal cells to inactivated, non-cytolytic HSV that is generated by interaction between active HSV and tobacco-related chemical carcinogens in the oral cavity of tobacco users, may induce molecular biological changes of the cells. These changes may ultimately be responsible for cell transformation, since the inactivated virus is able to produce viral proteins that might possess oncogenic potential.

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=국문초록=

발암성 화학물질들이 Herpes Simplex Virus의 복제, 세포융해, DNA 합성 및 단백질 합성에 미치는 효과

고려대학교 의과대학 약리학교실

천 연 숙

Benzopyrene (BP), 7,12-dimehyl benzanthracene (DMBA), nitrosomethyl urea (NUMU) 및 nicotine과 같은 발암성 화학물질들이 바이러스 감염된 vero 세포의 단층 세포 배양에서 I형 단순성포진 바이러스 (HSV-1)의 복제, 세포융해, DNA합성 및 단백질 합성에 미치는 효과를 관찰하였다.

1. BP와 DMBA는 HSV-1의 복제와 세포융해작용을 유의성있게 억제하였으나 nicotine과 NMU는 별로 억제하지 않았다.

2. 모든 발암성 화학물질은 바이러스의 DNA합성을 억제하지 못하였지만 새로 합성되는 후손 바이러스 DNA로 부터 표현되는 gamma 단백질의 표현은 BP와 DMBA에 의해서 현저하게 억제되었다. 그러나 모든 발암성 화학물질은 바이러스의 alpha 및 beta 단백질의 합성은 억제하지 못하였다.

이상의 결과로 보아 발암성화학물질이 존재하고 있는 배지내에서 새로 합성되는 바이러스의 DNA로 부터 표현되는 gamma 단백질의 결함이 있음을 알 수가 있었으며 이같은 개념은 발암화학물질의 존재하에서 바이러스의 DNA와 단백질이 거의 정상적으로 합성됨에도 불구하고 바이러스의 복제가 일어나지 않는다는 사실이 뒷받침해주고 있다.