International Immunopharmacology 24 (2015) 173–181

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Immunomodulatory effect of diethylcarbamazine citrate plus filarial excretory–secretory product on rat hepatocarcinogenesis

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ARTICLE INFO

Article history:
Received 17 July 2014
Received in revised form 6 November 2014
Accepted 1 December 2014
Available online 12 December 2014

Keywords:
Diethylcarbamazine citrate
Immunomodulation
Excretory–secretory product
Rat
Hepatocarcinogenesis

ABSTRACT

Diethylcarbamazine citrate (DEC) had a significance in anti-filarial chemotherapy, while excretory–secretory product (ES) is released from adult filarial females. The target of the current study was to examine the immunomodulatory effect of DEC, *Setaria equina* ES or a combination of them on rat hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DEN). *In vitro* effect of combined DEC and ES or ES alone on lipopolysaccharide (LPS)-stimulated rat peripheral blood mononuclear cells (PBMCs) was tested through IFN-γ assay in culture supernatants. In addition, single or repeated doses of DEC, ES or DEC + ES have been applied in white albino rats to test the effect on HCC. Levels of IFN-γ and anti-ES IgG antibodies in rat serum were assayed using ELISA. Hemolytic complement activity (CH_{50}) was determined in serum while the concentration of nitric oxide (NO) was assayed in liver tissue. The infiltration of NK cells as well as the expression of MHC Iproliferating cell nuclear antigen (PCNA), inducible NO synthase (iNOS), Bcl2 and p53 were determined using immunohistochemistry. There was a dose-dependent increase in IFN-γ after *in vitro* exposure to DEC + ES. Repeated ES doses increased NO concentration ($p < 0.05$) and expression of iNOS but reduced CH_{50} ($p < 0.001$), while repeated DEC + ES doses could increase anti-ES IgG ($p < 0.01$), IFN-γ level ($p < 0.05$) and NK cell infiltration. The same treatments could also reduce the expression of MHC I expression, PCNA, Bcl2 and p53. This study has shown immunomodulatory and protective effects of DEC + ES repeated doses on rat HCC.

1. Introduction

DEC has been known as one of the piperazine derivatives (N,N-diethyl-4-methyl-2-piperazine) which has a significance in anti-filarial chemotherapy [1]. It has also been known for its efficacy to eradicate Bancroftian filariasis in Egypt and other countries in the world [2,3]. The mode of action for DEC was postulated as indirect on the parasite through inhibition of arachidonic acid metabolism in microfilariae and in host endothelial cells leading to an increased host innate immunity [4]. DEC was postulated as an activator for NK-cell activity associated with increased IFN-γ and IL-2 release after treatment to filarial infection [5]. Furthermore, DEC was able to decrease the load of human immunodeficiency virus in co-infected patients with filariasis [6]. This could give an indication for potent DEC action during filarial infection. ES is released along with microfilariae from adult female worms. Exposure of mice to ES material resulted in an immunosuppression while treatment with DEC could initially potentiate the immune response followed by immunosuppression [7]. An immunomodulatory action of DEC after treatment to *Setaria equina* infected rats has been observed [8]. This reversed effect on immune system was revealed by an increase in infiltrated leukocytes, cellular adherence and microfilaricidal effect. Recently, *S. equina* ES was an antioxidant against rat HCC, while DEC could modulate such effect when combined with it [9].

The target of the current study was to examine the immunomodulatory effect of DEC, *S. equina* ES or a combination of them on the pathogenesis of rat HCC induced by DEN as a carcinogen for liver cancer. The test included either a single dose before induction of carcinogenesis or repeated doses with DEC, ES or DEC + ES. Released IFN-γ from rat PBMCs and serum IFN-γ were assayed. In addition, levels of NO in liver tissue and IgG in serum were shown. The complement activity in serum was determined. Infiltration of NK cells as well as expression of MHC I, PCNA, iNOS, Bcl2 and p53 proteins in liver tissue were investigated using immunohistochemistry.

2. Materials and methods

2.1. Collection of S. equina adult female and preparation of ES

Eighty adult female worms were collected from the peritoneal cavity of equines during slaughtering for lion feeding. The worms were collected in peritoneal fluid and transferred to laboratory. Thereafter, worms were washed and incubated at 37 °C for 6 h [10]. The prepared ES material was concentrated 4–5 times, dialyzed against 100 mM phosphate buffer pH 7.6 at 4 °C and stored at −70 °C until use. Protein contents were estimated [11].

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http://dx.doi.org/10.1016/j.intimp.2014.12.004
1567-5769/

2.2. Isolation of rat PBMCs and IFN-γ assay

PBMCs were isolated from the peripheral blood of five male white albino rats by following a method originally described [12]. Briefly, blood was collected into heparinized tubes and mixed with an equal volume of Hanks' buffered salt solution (HBSS). Aliquots (20 ml) of diluted blood were then layered onto cushions of Ficoll-Hypaque (7.5 ml; Sigma) and centrifuged at 400 *g* for 20 min at 22.5 °C. PBMCs were aspirated from the gradient interface and washed three times with HBSS at 300 *g* for 10 min at 22.5 °C. Single cell suspensions were prepared in RPMI 1640 medium (Sigma) containing 10% heat inactivated fetal calf serum and supplemented with L-glutamine (0.3 mg/ml), penicillin (50 IU/ml) and streptomycin (50 μg/ml). Their viability, as judged by Trypan blue exclusion was found to exceed 95%. Aliquots (0.5 ml) of cell suspension (2×10^6 cell/ml) were dispensed into 24-well plates. The cells were incubated for 2 h with ES (50 μg/ml), DEC (Sigma, St. Louis, USA) at different concentrations (0, 50, 100, 200 and 400 μM) or combinations of later concentrations with ES. The cells were then stimulated with 10 μg/ml LPS (Sigma) and left for 24 h at 37 °C and 5% CO_2. The cells were centrifuged at 300 *g* for 10 min and the supernatants were harvested to measure the level of secreted IFN-γ using a rat IFN-γ ELISA kit (Koma Biotech Inc., Seoul, Korea). The procedure was performed according to the instructions provided with the kit.

2.3. HCC model and regimen of treatment

The experimental HCC was initiated by DEN and promoted by 2-acetylaminofluorene (2-AAF; Sigma), according to the protocol described [9]. HCC was evaluated by histological observations for the rat liver. Eighty male white albino rats (180–200 g weight, 2 months of age) were randomly allocated into eight groups of 10 animals each and were housed in a well-ventilated environment. All animals received professional humane care in compliance with the guidelines of the Ethical Committee of Science Faculty, Beni-Suef University, Egypt. The first group was a negative control while other groups were induced for HCC. For the induction of HCC, rats were injected intraperitoneally with a single dose of DEN (Sigma) dissolved in water (100 mg/kg body weight). They also received *via* gavage a solution of 2-AAF (20 mg/kg body weight) in 1% Tween 80 (Sigma) on days 7, 8 and 9 after DEN injection for promoting carcinogenesis. One of these seven groups acted as a positive control group because it was only exposed to DEN and 2-AAF without any treatments. Rats in the negative control group (G1) were given an i.p. injection of the same volume of water and gavage administration of 1% Tween 80 in saline as those in the positive group (G2). The other six rat groups were divided into three for single dose treatment and three for repeated dose treatment. Three groups (G3, G4, and G5) were pretreated i.p. with 100 mg/kg DEC [13] in saline, 100 μg ES [14] in Tyrode's solution and combination of both two days before DEN injection. The other three groups (G6, G7 and G8) were pretreated as in the first three groups but also post-treated with the same materials. Post-treatments were given per month for a period of 4 months. G1 was also injected with saline and Tyrode's solution at the time of pre- and post-treatments.

2.4. Serum IFN-γ and liver homogenate NO assays

After one week from the last injection, animals were euthanized where serum and liver tissue samples were taken. Collected blood samples were centrifuged at 1500 *g* for 20 min at 4 °C to obtain serum. Serum IFN-γ was measured as illustrated above.

Liver homogenates were centrifuged at 1000 *g* at 4 °C for 20 min and supernatants were taken. Aliquots were prepared and used for determination of NO. Nitrite concentration in the supernatants was assayed in a microplate by the Griess reaction [15]: 100 μl of the homogenate supernatant was added to 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine hydrochloride in 2.5% H_3PO_4), then incubated for 10 min at room temperature, and absorbance was measured at 530 nm using an ELx808 ELISA reader (BioTek, Winooski, USA). Nitrite concentration was calculated with reference to a standard curve obtained using $NaNO_2$.

2.5. Complement activity

The hemolytic complement activity (CH_{50}) in rat sera was determined [16]. In short, rat serum was 1:4 prediluted in Veronal Buffered Saline (Sigma; VBS^{2+}; barbital sodium 5 mM-buffered saline 0.15 M pH 7.4, containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+}). Duplicate serial dilutions were made in a total volume of 200 μl. An equal volume of sensitized sheep erythrocytes with Amboceptor (anti-sheep erythrocytes; Siemens, Munich, Germany) in VBS^{2+} was added (final volume is 400 μl). Tubes were incubated at 37 °C for 30 min in a water bath and centrifuged at 1500 *g* for 5 min to sediment the RBCs. One hundred microliters of supernatant was transferred from each tube to a well in a 96 well flat bottom plate and 100 μl of distilled water was added to each well. Absorbance was read at 540 nm using an ELISA reader. Blank absorbance (spontaneous lysis) and total lysis (100% lysis) were calculated. The percentage of lysis (%) was calculated using the following formula:

$$\%\,\text{lysis} = \frac{\text{OD}_{540}\text{test} - \text{OD}_{540}(\text{Blank})}{\text{OD}_{540}(\text{total lysis} - \text{OD}_{540}(\text{Blank}))} \times 100.$$

The percentage lysis (vertical axis) was plotted *versus* the serum dilution on the horizontal axis. The dilution required for 50% hemolysis for the control and test serum was calculated.

2.6. Immunohistochemistry

Liver tissue segments were frozen in liquid nitrogen and cut at 5 μm sections. The slides were then incubated in 3% H_2O_2 for 10 min at room temperature to block the endogenous peroxidase activity. Slides were treated with 1.5% normal serum obtained from the same species in which the secondary antibody was developed for 30 min to block non-specific staining. Subsequently, slides were incubated with monoclonal antibodies against rat PCNA, NK cells (CD161), MHC I (RT1A), iNOS, p53 and Bcl2 (1:100; AbD Serotec, New York, USA) for overnight at 4 °C. Then slides were treated with a biotin-conjugated secondary antibody for 10 min followed by incubation with peroxidase-conjugated streptavidin for 10 min at room temperature according to the instructions of Histostain®-Plus 3rd Gen IHC Detection Kit (Life Technologies, California, USA). All the above steps were followed by washing in Tris buffer (pH 7.4) for 3 times. Immunolabeling was detected by incubation with 0.06% diaminobenzidine (Sigma) dissolved in tap water containing 0.01% H_2O_2 for 3–5 min, followed by washing and staining with Mayer's hematoxylin. Quantitative determination for the positively stained NK cells in the liver tissues was performed by counting at ×400 magnification in similar-sized areas of different sections and expressed as mean ± S.E. [17].

2.7. Detection of rat IgG levels against ES

Maxisorp flat-bottom 96-well microtiter plates (Nalge Nunc International, Denmark) were coated with 100 μl per well of coating buffer, pH 9.6, containing 1 μg/ml of ES and incubated overnight at 4 °C. The antigen-coated plates were washed three times with wash buffer (PBS containing 0.1% Tween 20). The wells were blocked with 200 μl of blocking buffer (5% fetal calf serum in wash buffer) for 3 h at 37 °C. After washing, the plate was blotted dry on a paper towel. The test sera of G2, G3, G5, G6 and G8 individual rats were diluted at 1:200 in wash buffer and added (100 μl/well) into coated wells. Incubation of sera was for 2 h at room temperature. After washing three times, the coated wells were incubated for 1 h at room temperature with 100 μl/well diluted peroxidase-labeled goat anti-rat IgG (KPL, Maryland, USA) at 1:2000