DMBA Induction Increases H-Ras Gene Expression and Decreases CD8 Count in Sprague Dawley Rats

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ABSTRACT

Introduction: DMBA is carcinogenic-immunosuppressant. This study was conducted to investigate the immunosuppressant effect of dimethylbenzanthracene (DMBA) on H-Ras expression, hemogram, and CD8 count I Sprague Dawley (SD) rats. Methods: We divided the test animals into three groups. The normal group received standard food and drink. We induced the DMBA group with 10x20mg/kg BW DMBA for five weeks. The solvent group received standard feeding and corn oil solutions. We conducted dissection and data retrieval in week 27. The immunosuppressant effects of DMBA are measured by number leukocytes and CD8CTL. The number of CD8 is determined by the flow cytometer. The H-Ras gene expression is assessed using densitometry after PCR. The mean intergroup difference was calculated using ANOVA. Result: The results showed that DMBA administration increases H-Ras gene expression and decreases CD8 count ($p<0.05$). 10x20 mg/kg BW DMBA administration decrease leukocytes and platelet ($p<0.05$). Conclusion: A dose of 10x20 mg/kg BW DMBA decreased CD8, leukocytes, and platelet number, while it increased H-Ras gene expression in SD rats.

CCS Concepts

• **Applied computing → Life and medical sciences → Systems biology**

Keywords

Keywords immunosuppressant; DMBA; CD8; H-Ras Gene

1. INTRODUCTION

Breast cancer is a public health problem in Indonesia and the world. One of the factors associated with an increased incidence of breast cancer in Indonesia is the high carcinogenic air pollutants, including smoke from cigarettes, motor vehicles, industries, and households [1, 2, 3]. These smoke sources contain carcinogenic polycyclic aromatic hydrocarbons, one of which is 7.12 dimethylbenzanthracene (DMBA) [4,5]. In addition to being genotoxic, DMBA is also immunotoxic [6]. Cancer cell formation due to exposure to DMBA is a complex phenomenon, as it involves the immune system and various enzymes in the body's metabolic system [7, 8, 9]. DMBA can trigger the formation of breast, skin, blood, oral, and pulmonary cancer through either the mechanism of DNA mutation by forming DNA adduct or chronic inflammatory mechanism [10, 11]. DMBA is metabolized by cytochrome, and epoxide microsomal hydrolyzes enzymes into 7.12-dimethylbenzanthracene-3,4-diol-1,2, epoxide (DMBA-DE), the ultimate carcinogen that is both genotoxic and immunotoxic [12, 13]. DMBA-DE binds to DNA to form DNA adducts with p53 and H-Ras genes as carcinogenesis initiators [14]. The formation of DNA adducts in the p53 gene by DMBA-DE causes inactive repair functions and the mechanism of proapoptosis against neoplasms. Conversely, the formation of DNA adducts in the H-Ras gene by DMBA-DE activates proto-oncogene, neoplasms undergo hyperproliferation. [14, 15, 16]. p53 is a main tumor suppressor protein that has an essential role in anti polycyclic aromatic hydrocarbon-induced carcinogenesis. In vivo study, DMBA-DE has been shown to suppress bone marrow activity [17], inhibit T cell activity [18], toxic agents to splenocytes [7, 8], and inhibit cytokine activity and stimulator IFN-γ gene (STING) [19]. These conditions undermine the immune system and allow neoplasms to develop into malignant tissues [20].

Cytotoxic lymphocyte (CD8) is essential in stemming the development of neoplasms into tumor tissue. The immune system's inability to recognize cancer cells as non-self and to eliminate it is the beginning of damage to the body defense system in providing resistance to carcinogenesis [21, 22]. Inadequate host immune response to neoplasms, especially by cytotoxic T cells (CD8) to eliminate cancer cells, will increase cancer-induced morbidity and mortality [23]. Cytotoxic T cells (CTL) act as effectors in the destruction of malignant neoplasms directly or indirectly by producing various anticancer cytokines as carcinogenesis inhibitors [24-28]. The p53 gene plays an essential role in the activation and effectiveness of CTL performance in inhibiting carcinogenesis. P53 have been proven to be effective in increasing the cytotoxic lymphocyte activity in eliminating neoplasms and inhibiting carcinogenesis by activating the stimulator IFN-γ I gene (STING) [29-33] and increasing granzyme activity [34-36].

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It has also been shown to increase lymphocyte proliferation [37-41] and macrophage phagocytosis activity [42] and proven as useful as chemopreventive in colonic cancer [43]. The data from this study show that DMBA seems to be effective as immunosuppressant-carcinogenesis in DMBA-induced SD rats through increase Ras gene activity and decrease cytotoxic T cell numbers.

2. MATERIAL AND METHOD

2.1 Animal Experimental Design

This study was an experimental laboratory with a control group. The test animals used were Sprague Dawley (SD) aged 3-4 weeks, divided by eight groups. The study was conducted at the animal breeding and experimental unit of GadjahMada University. We used 7,12 dimethylbenzanthracene (DMBA, Sigma-Aldrich) for inducing carcinogenesis. The study protocol has been reviewed and declared ethically viable by the animal research ethics committee of Gadjah Mada University, Yogyakarta, Indonesia (No 043/KEC-LPPT/II/2019).

2.2 Materials and Equipment

The FITC CD8 antibody monoclonal for flow cytometry is obtained from Abcam. Hematoxylin-eosin and Giemsa are used for the painting of histopathologic preparations. Devices used for gene activity examination include PCR machine (Thermocycler Biometra, Göttingen, Germany), centrifuge (Heraeus Biofuge fresco Kendro, Hanau, Germany), Eppendorf tubes,micropipettes, electrophoresis (SE 260 Mini-Vertical electrophoresis-unit GE Healthcare, Munich, Germany), Mini-shaker (MS 2 IKA, Wilmington, USA),densitometry, and spectrophotometer. Materials for PCR include DNA markers (catalog No. 1721933, Roche Diagnostics GmBH), Trizol (catalog No. 10503-027, Invitrogen USA), RTG PCR and RTG-RT PCR kit (pure tag Ready-To-Go RTPCR beads , catalog No. 279557-01), room temperature beads with stabilizers, BSA, dATP, dCTP, dGTP, dTTP (catalog No. 27-9557-02), pure tag DNA polymerase, (catalog No.27-9558- 01), dNTP (catalog No. 27-9559-01) GE Healthcare, UK), first-strand cDNA synthesis kit for RT-PCR (Roche), loading buffer, agarose, and other chemicals. The p53 and H-Ras DNA primers are presented in Table 1.

Table 1 Primary list for RT-PCR[44]

Gen	sequencing	bp	T annealing	temperature	link	Ref
H-Ras exon 1	F:5-TGG TTT GGC AAC CCC TGT AGA-3 B:5-AGTGGGATCATACTCGTCCAC-3	305	62	72	49	45
b-Actin	F:5-AAGAGAGGCATCCTCACCCT-3 B:5-TACATGGCTGGGGTGTTGAA-3	815	55	72	33	46

Note F: forward; B: backward;

The female Sprague Dawley (SD) rats aged 3-4 weeks weighing from 100 to 140 grams (uniform age and weight) are obtained from the Gadjah Mada University Biological Sciences Laboratory. The test animals were kept in a 50 x 30 x 20 cm individual-sized iron cage, fed 528 pellets, and fed moderately.

Ninety-six of a 2-week-old female SD line were treated in a 50 x 30 x 20 cm individual iron cage. Rats are kept under good animal testing standards in terms of cage conditions, foods, and drinks. Before the study, test animals were put in a maintenance cage for one week for adaptation. During the study, the rats were weighed every week to measure weight development. Breast cancer mouse modeling was performed by administering DMBA with the procedure similar to previous researchers but with modification. The 5-week-old mice were given DMBA solution in corn oil at a dose of 20 mg/kg BW per-orally. The DMBA initiation is repeated twice a week, making it ten times in total [44]. After the adaptation period, we divided the test animals randomly into three groups. Group I is the normal group. The animals in this group only received standard feeding during the test. Group II is the DMBA group. It received corn oil for 14 days before being induced with 10x20mg/kg BW DMBA for five weeks. Group III is the solvent control group. It received 100 mg/kg BW/day corn oil and was induced with DMBA in addition to standard feeding and drinking. The corn oil is administered in the same preparation and manner as the other groups. All test animals received 10x20 mg/kg BW DMBA orally within five weeks starting from the 3rd week of treatment, except for the normal group and the solvent control group.

2.3 Examination of Immunosuppressant Effects of DMBA

The DMBA-immunosuppressant effects are measured by hemogram profile [8,45]. An examination of the hemogram profile is done with a hemo-analyzer. Blood collection is done through the orbital vein. The leukocyte count of the DMBA group was compared with the normal group. The leukocyte count of the treatment group was then compared with the leukocyte count of the DMBA group.

2.4 Examination of H-Ras Gene Expression

Isolation of RNA from the tissue and the formation of cDNA; RNA isolation from mammary and hepatic tissues was performed using an RNAzol kit with the procedure as recommended by the company. The 20 µL of isolated RNA solution was used for cDNA formation. The cDNA formation was done using the First-Strand Synthesis Kit for RT-PCR version 09 (AMV, Roche). The 3 µg RNA concentration in 30 µL diethylpyrocarbonate (DEPC) 0.1% was homogenized with the vortex in a microtube, and then incubated at 65° C for 10 minutes. The microtube was then inserted into the ice for 2 minutes, and then the RNA was inserted into the first strand synthesis reaction tube containing two white cotton balls. Primary oligo (dT) 5'-GTA at cga ata act ata ggg cac gcg tgg tg gcc cgg gct ggt ttt ttt ttt ttt t-'3 with 1 µg /3µL concentration added into the reaction, then left for 1 minute. Microtube was incubated at 37° C for 1 hour, and then SDW 50 µL was added to the cDNA formed.

2.4.1. Performing RT-PCR using RTG RT-PCR Kit

The RT-PCR material preparation procedure was as follows: (1) up to 2 µL primer was added into RTG RT-PCR; (2) cDNA template was added at a volume according to calculation; (3) up to 25 µL

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H2O DNase was added; (4) The solution was then homogenized by beating the lower end of Eppendorf smoothly; (5) 25 µL mineral oil was added to homogeneous solution; (6) the Eppendorf was then opened and the test material was ready for DNA amplification. DNA amplification of the test material was carried out with the GeneAmp® PCR system thermal cycler. The prepared Eppendorf tube was fed into the PCR device, and the temperature and number of cycles were adjusted as required. The denaturalization process was done for 5 minutes at 95oC. The overall cycle, link, and temperature settings are shown in Table 1.

Electrophoresis product of RT-PCR test material with agarose gel is carried out by experienced technicians. The preparation procedure for 1.5% agarose is as follows: (a) 0.50 grams of agarose was put into Erlenmeyer; (b) 25 ml of 0.5X TBE was added; (c) after that, it was put into the oven for 150 seconds; (d) afterwards, 2 µL ethidium bromide was added; (e) then, after homogeneity, it was poured into the gel mold; (f) wait for 1 hour before the gel was used in order for the gel to harden. The hardened gel is then fed into the electrophoresis chamber. In the next stage, the PCR DNA analyte was inserted into the electrophoresis well with the following procedure: (a) 10 µL of the PCR solution was piped and inserted in sterile Eppendorf tube; (b) 1 µL loading buffer was added and homogenized by beating the lower end of the tube; (c) 2% agarose gel was introduced into the electrophoresis apparatus and 0.5X TBE was added until the agarose gel completely immersed; (d) The PCR solution was piped and inserted in the agarose; (e) the electrophoresis device was set to 100 volts voltage and 43 minutes of elution time; (f) if the buffer spot had reached the upper limit, the electrophoresis apparatus was turned off and the gene expression was seen under the UV light. The expression of a visible gene with a UV lamp is then documented for analysis.

2.4.2. Semi Quantification of H-Ras Gene Expression by Densitometry

Semiquantitative analysis of gene expression was performed with the aid of densitometry tools. Documentation photos of gene expression bands after electrophoresis were printed in black and white in postcard size. The difference in the size and thickness of the DMBA band (bend) image of PCR results from the gene is then read by densitometry at a visible wavelength (420 nm). The magnitude of gene activity is indicated by the sheer size or thickness of the bender image and by densitometry reading as the area under the curve (AUC). Comparison of DNA tape AUC and DNA bands AUC of the b-actin gene is referred to as gene activity.

2.5 CD8 T Cytotoxic T Lymphocytes Measurement

The immune response of anti-carcinogenesis surveillance is made by observing the number of cytotoxic lymphocytes (CTL) or CD8. Calculation of the number of CD8 lymphocytes types was performed by the flow cytometer (Beckman Coulter FC500). Blood taken from the conjunctiva of the test animal's eye was put into a vacutainer tube containing an anticoagulant. The flow cytometer examination was done with the following procedure: (i) the specimen was piped into a 50 µL falcon tube; (ii) 10 pL tritest reagents CD FITC / CD per CP was added; (iii) it was then mixed using the homogeneous vortex mixer and incubated for 15 minutes at 20-25° C in the darkroom; (iv) 50 pL of I0x FACS solution of cell lysis was diluted with 450 pL aqua dest and they were mixed homogeneously; (v) After the incubation, 450 pL of the diluted FACS reagent was added to the specimen (lx); (vi) They were homogeneously mixed and then incubated for 15 minutes at 20-25° C in dark room; (vii) Afterwards, the analysis was done using FACS flow cytometry.

2.6 Data Analysis

Statistical analysis of the study results was accomplished using different methods for different data types. Data on the number of mice containing breast tumors or nodule (incidence) were presented descriptively, on weight gain with ANOVA followed by Tukey test, and on the number of tumor multiplicity among the groups with Non-Parametric Kruskal Wallis statistical test with 95% confidence level followed by Man Whitney Test. The results of histopathological examination of nodules and mammary tissues were presented descriptively. Examination results of H-Ras gene expression were presented descriptively as the ratio of H-Ras gene expression to actin gene expression. Relative activities of H-Ras genes on actin genes were analyzed with mean difference test (ANOVA) with a 95% confidence level. The number of cytotoxic cells was presented descriptively and then tested with one way ANOVA with a 95% confidence level.up.

3. RESULTS AND DISCUSSION

3.1 Hemogram

The results of the examination of the effect of DMBA induction on hemograms are presented in Table 2. The results showed that the induction of DMBA 10x20 mg/KgBW reduced the number of leukocytes and the number of platelets. The average number of leukocytes and platelets. The DMBA group was lower than the healthy group ($p < 0.05$), but clinically within the normal range. DMBA induction did not affect the number of erythrocytes, hemoglobin levels and hematocrit ($p > 0.05$).

Table 2. Effect of induction of 10x20 mg/kg BW of DMBA for five weeks on the number of leukocytes, erythrocytes, hemoglobin, hematocrit, and SD rat platelets

Characteristic	DMBA	Normal	Solvent	Total	P value
Leukocytes (x106/dl)	5.70±2.15	11.83±0.41	11.78±0.41	8.44±3.96	0.00
Erythrocytes (x106/dl)	6.17±1.98	6.72±0.53	6.68±0.71	6.69±1.59	0.07
Hb (%)	12.18±3.49	12.26±0.56	12.03±0.72	12.76±3.09	0.08
Hct	36.96±9.13	34.10±1.640	33.85±1.72	34.47±8.01	0.06
Platelet (x103/dl)	.768.40±0.28	1055.00±0.001	1058.30±0.03	797.50±0.42	0.00

3.2 The Result of H-Ras Gene Expression Examination

The results showed that DMBA induction increased H-Ras gene expression (Table 3). The normal group H-Ras gene expression was lower than the DMBA group. Table 3 presents the relative activity of the H-Ras gene nodules of mammary tissues. The activity of the H-Ras gene of the DMBA group (3.28 ± 0.10) was higher than that of the normal group (0.26 ± 0.13) ($p < 0.05$). ($p < 0.05$). The results

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show that DMBA induction increases the relative activity of the H-Ras gene.

Table 3. Relative activity of the H-Ras gene in DMBA-induced primary mice 10x20 mg/kg BW for five weeks. Tissue retrieval grazed at week 27 of the experiment

Groups	N	Gene relative activity to B-Actin
		H-Ras gene (mean±SD)
Normal	12	0,26±0,09*
DMBA	12	3,28±0,62
Solvent	12	0.28±0.34

Note: *=p<0.05 to the DMBA group

The activity in the DMBA group (3.28 ± 0.62) was higher than that in the healthy group (0.18 ± 0.02) (p <0.05). The results prove that DMBA induction increased H-Ras gene activity.

3.3 Cytotoxic T Cell (CD8) Numbers

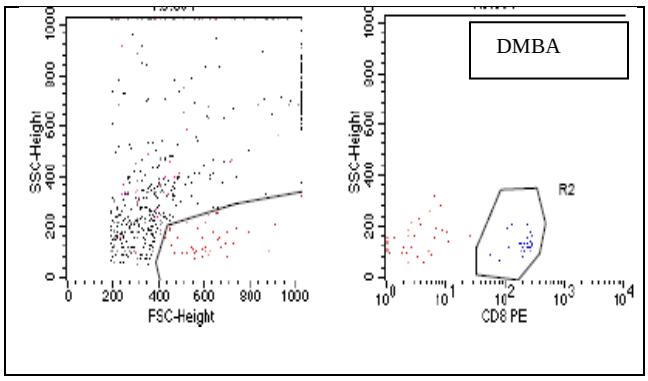
The anti-carcinogenesis immune response of DMBA-induced primary rats with BCSO administration was observed using the cytotoxic lymphocyte count (CD8) parameter. DMBA induction was shown to decrease the absolute number of CD8 cytotoxic T lymphocytes. The result of a flow cytometer examination of CD8 cytotoxic T lymphocyte number for the healthy group was 580 cells/ml, and the DMBA group was 137±18.48 cells/ml. The results of the flow cytometry test to determine the total number of CD8 cytotoxic lymphocytes are shown in Table 4.

Table 4. CD8 count of 10x20 mg/kg BW DMBA-induced SD Rat. Blood sampling was performed at week 27

Groups	n	CD8 absolute number	CD8 percentage
Normal	12	580.00±66.63*	10.02±1.54*
DMBA	12	137.00±18.48	4.39±2.38
Solvent	12	668.33±390.56*	8.82±2.25*

Note: *=p<0.05 to the DMBA group

Table 4 and figure 3 show that DMBA induction decreased the amount of CD8 cytotoxic T lymphocytes.



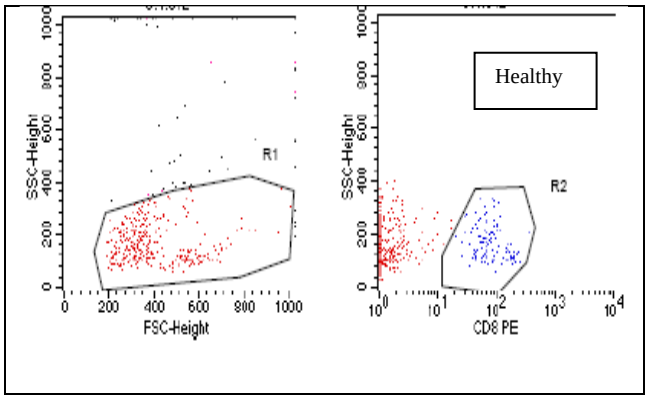


Figure 1. Dot plot lymphocyte CD8 count of SD rats receive induction DMBA of 10x20 mg/kg BW, 2x / week.

The results showed that the most significant percentage of CD8 cytotoxic lymphocytes was in the normal, and finally the DMBA group

3.4 Discussion

The results show that DMBA induction was shown to increase the risk of animal mortality, and 10x20 mg/kg BW DMBA administration also proved to cause carcinogenesis. These results are consistent with previous research. The results of prior research have shown that DMBA metabolite (DMBA-DE) forms DNA adduct in p53 and H-Ras genes and initiates carcinogenesis. This study has been demonstrated that DMBA induction can decrease p53 gene expression and increase H-Ras gene expression [46-48] (Hidayati et al., 2019). Breast cancer neoplasms express new molecules of malignant determinants as a result of gene dysregulation [4.10]. Gao et al. (2007) have proven that DMBA metabolites (DMBA-DE) successfully formed DNA adduct in the p53 gene resulting in immune toxicity [8]. Mousa et al. (2004) and Mbarek et al. (2007) have proven that Black Cumin Seed extract is antigens [49,50]. BCSO mechanisms in inhibiting carcinogenesis caused by DMBA induction include the activation of NFKb pro-apoptosis pathway via the p53 gene. Sethi et al. (2008) have demonstrated that thymoquinone activates the NF-kB pathway and thus suppressing the anti-apoptotic gene and enhancing apoptosis [51]. BCSO has also been shown to restore wild-type p53 genes that are damaged by genetic stress due to DMBA-DE exposure so that the p53 regulating function is reactivated [49].

These study results have revealed that DMBA induction can decrease the number of CD8 cytotoxic lymphocytes.t. In addition to causing changes in the expression of various surface protein molecules, genetic dysregulation in neoplasms can also lead to transcriptional and translational disorders of intracellular protein molecules that are identified as non-self and immunogenic [50]. DMBA-DE has also been confirmed to be immunotoxic and suppressing bone marrow hematopoietic activity [17]. Cellular injury due to genetic stress and protein molecules of mutant gene products due to p53 genetic dysregulation can inhibit specific immune responses and activate inflammatory reactions [28]. Environmental factors play a significant role in carcinogenesis, neoplasms, and tumor tissue formation [52]. As the immune system weakens and the inflammatory reaction becomes excessive, cancer formation in the DMBA group is most successful [54,20]. BCSO administration can inhibit DNA damage caused by DNA adduct formation, prevent carcinogenesis, and improve specific immune

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responses through an increased number of cytotoxic T lymphocytes. These findings support the notion that the function of T-cytotoxic cells is to immunosurveillance and destroy cells containing mutant genes that cause tumors or are associated with malignant tumors [55]. With the help of perforin and granzyme, CD8 cytotoxic lymphocyte cells perform lysis of mutant cells expressing the foreign peptide displayed by MHC class I [56]. The activity of lysis or apoptosis of cancer cells by CD8 cytotoxic cells with granzyme intermediates turned out to involve the p53 gene. The granzyme released by cytotoxic lymphocytes proves to stimulate p53 gene activity [29]. The concept of immunosurveillance is based on the assumption that the immune system has a role in preventing and limiting tumor growth [57], including, in this case, carcinogenesis of breast cancer due to exposure to DMBA chemical carcinogens. One of the cellular components that play a crucial role in immunosurveillance against carcinogenesis is CD8 cytotoxic T lymphocytes. Increased immunosurveillance activity was shown by CD8 cytotoxic lymphocytes. Antioxidants and herbal immunomodulators can inhibit the immunosuppressant effects of DMBA and other aryl hydrocarbon carcinogens. Black seed has been proven to have antioxidative and immunomodulatory effects both preclinical and clinical [58-62].

4. CONCLUSION

The conclusion of this study is that the induction of DMBA decreased the number of leukocytes, platelet, and CD8 cytotoxic T cells. The induction of DMBA also increased the H-Ras gene expression in SD rats.

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