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The Relationship between Area of Necrosis with Degree of Lymphocytes Infiltration in Breast *Rattus norvegicus* Induced by DMBA

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Abstract

Breast cancer is one of the leading causes of death in women in the world. According to WHO, the incidence of breast cancer is about 2.1 million. The high mortality rate is often influenced by various reasons, one of which is the prognosis in patients. The level of lymphocyte infiltration in tumor cell tissue has been shown to correlate with a better prognosis in patients, while necrotic tissue found in breast cancer often shows worse prognostic value. To determine the relationship between the area of necrosis with the degree of infiltration of lymphocytes in the breast of *Rattus norvegicus* induced by DMBA. Method: True experimental study with post-test only controlled group design method using 40 Wistar strains of female *(Rattus norvegicus)* mice 12-13 weeks old. The technique for taking uses simple random sampling. Examining 2 variables, namely the dependent variable area of necrosis and the independent variable degree of lymphocyte infiltration. The treated group is injected with subcutaneous DMBA 10 mg/Kg BW between the two lower nipples during the middle of the fifth week with a three-week interval of administration to reach 14 times the total administration. Cancer tissue is taken in the fifth week. Data are analyzed by the Spearman test. Results: This study shows no relationship (P> 0.05) between the area of necrosis with the degree of lymphocyte infiltration. Conclusions: There is no relationship the area of necrosis with the degree of lymphocytes infiltration in breast *Rattus norvegicus* Induced by DMBA.

Keywords: Infiltration, Lymphocytes, Necrosis

1. Introduction

Cancer is one of the leading causes of death worldwide. Breast cancer is the most common cancer in women. In 2020, an estimated 685,000 women died from breast cancer, accounting for 15% of all cancer deaths among women.[1] Tumor-infiltrating lymphocytes (TILs) play an important role in mediating the chemotherapy response and improving clinical outcomes in all subtypes of breast cancer. In addition, the degree of lymphocytic infiltration in tumor cell tissue has been shown to correlate with a better prognosis in various tumor types as well as predict appropriate therapy in breast cancer.[2]

One way of identifying necrosis in tumors has prognostic value in treatment planning because necrosis is associated with an aggressive form of cancer and an unfavorable outcome.[3]

Research with the manufacture of animal models of breast cancer has been carried out using the induction of carcinogens. One of the carcinogens that are often used is 7,12 *dimethylbenz (α) anthracene* (DMBA) because it has a higher potential and is more stable as a carcinogen for the manufacture of animal models of cancer.[4] Ohtani study (2007) concludes lymphocyte infiltration is very common in early-stage colorectal cancer and rarely in late-stage colorectal cancer. The

higher stage of cancer and the lower level of lymphocyte infiltration is what causes the high risk of micrometastasis and the risk of colorectal cancer recurrence which will then lead to an improvement in the patient's prognosis.[5]

In this study, we aim to know the relationship between the area of necrosis with the degree of lymphocytes infiltration in breast *Rattus norvegicus* induced by DMBA.

2. Method

2.1 Research Design

This study design is an experimental laboratory using a true experimental approach of post test only controlled group design with simple random sampling.

2.2 In Vivo

The experimental animal in this study use a female white rat *(Rattus norvegicus*) Wistar strain, about 12-13 weeks and 200-300 g body weight. The sample amounted to 34 individuals which were divided into 2 groups, they are (i) a negative control group (normal), (ii) a positive control group (DMBA induction).

2.3 Protocol for Making Breast Cancer in Model mouse induced by DMBA

Induction was carried out with 3 times inductions per week and was given up to 14 times totally. Induction was carried out at a dose of 10mg/kg BW with 1 ml corn oil as a solvent. The one-time dose is 2 ml. The injection is done subcutaneously between the two lower nipples.

2.4 Procedures for taking and making breast tissue preparations.

This was done by intentionally euthanizing/killing the rats using Ketamine 20-40 mg/kg BW intramuscularly, after the rats died, they were dissected, and the breast organs were removed after which they were fixed with a 10% neutral buffered formalin (NBF) solution. Preparations were made using the paraffin block method, then the preparations were stained using the hematoxylin-eosin (HE) method.

2.5 Histology analysis

Observation of the histology of the breast using a light microscope to see the composition of breast cells and to see histopathological changes such as the area of necrosis and the degree of infiltration of lymphocytes. Then, the photo was taken with Optilab mounted on an Olympus CX 31 light microscope.

The grade for the area of necrosis is an area of cell death in the tissue as evidenced by the presence of reddish necrotic tissue, does not take hematoxylin dye, and is often pale in color. Score 0 given if no necrosis; score 1 given if necrosis of focal area (<25% from the tumor); score 2 given if widespread areas of necrosis (25-75% from the tumor); score 3 given if almost necrosis area from the tumor (75-100%). The grade for lymphocyte infiltration use to find out the lymphocyte infiltration into a necrotic area, divided into mild (0-10%); moderate (20-40%); and severe (50-90%).[6,7]

2.6 Ethical Clearance

Performed with the approval of the Ethics Committee of Animal Research, Faculty of Medicine, Universitas Hang Tuah Surabaya.

2.7 Data Analysis

This study used two data analyses are univariate and multivariate. Univariate analysis with descriptive analysis. Multivariate analysis was performed with the Spearman test using IBM SPSS Statistics.

3. Results

The results of rat breasts histology induced by the carcinogenic substance DMBA can be seen in Figure 1 and Figure 2 below.

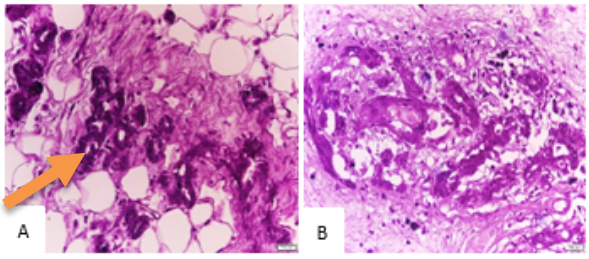

Figure 1. Histopathology of rat breast tissue *(Rattus norvegicus)* with 40x magnification of the objective lens. Description: A. Normal breast gland, B. Breast gland tumor.

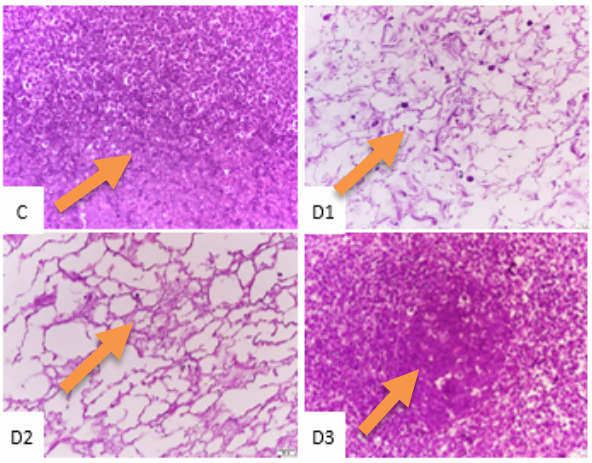

Figure 2. Histopathology of rat breast tissue *(Rattus norvegicus)* with 40x magnification of the objective lens. Description: C. Extensive area of necrosis, D1. Mild Lymphocyte Infiltration, D2. Moderate Lymphocyte Infiltration, D3. Severe Lymphocyte Infiltration.

3.1 Area of Necrosis

Based on Table 1 below, more than half of the sample (52.9%) was not found necrosis area.

Table 1. Histopathological view Area of Necrosis

No	Area of Necrosis (grade)	n	%
1.	No necrosis (0)	9	52,9
2.	Focal areas of necrosis (1)	5	29,4
3.	Wide area of necrosis (2)	3	17,6
4.	Almost all necrosis (3)	0	0,0
	Total	17	100

3.2 Degree of Lymphocyte Infiltration

Based on Table 2 below, almost half of the sample has severe lymphocyte infiltration (41.2%).

Table 2. Degree of Lymphocyte Infiltration

No	Degree of Lymphocyte Infiltration	n	%
1.	Mild	5	29,4
2.	Moderate	5	29,4
3.	Severe	7	41,2
	Total	17	100

3.3 Relationship Area of Necrosis with Degree of Lymphocyte Infiltration

The cross-tabulation between Area of Necrosis and Lymphocyte Infiltration Degree was divided into 4, namely no necrosis (0), focal area of necrosis (1), extensive area of necrosis (2), almost all necrosis (3) shown in Table 3. More than half of the mice did not have areas of severe lymphocytic infiltration necrosis. Less than half of the mice had focal areas of necrosis with the category of mild lymphocytic infiltration. Less than a quarter of the mice had large areas of necrosis with moderate lymphocytic infiltration.

Table 3. Cross-tabulation between Area of Necrosis and Lymphocyte Infiltration

No	Area of Necrosis	Degree of Lymphocyte Infiltration						n
		Mild		Moderate		Severe		
		n	%	n	%	n	%	
1	No necrosis	2	11.8	2	11.8	5	29.4	9
2	Focal areas of necrosis	3	17.6	1	5.9	1	5.9	5
3	Wide area of necrosis	0	0.0	2	11.8	1	5.9	3
4	Almost all necrosis	0	0.0	0	0.0	0	0.0	0
	Total	5	29.4	5	29.5	7	41.2	17

Statistical analysis test was used to determine the relationship between area of

necrosis and degree of lymphocyte infiltration. We use a statistical test of the relationship, namely the Spearmen analysis test because it is assumed that there is a relationship for ordinal-ordinal data types.

The results of statistical tests using Spearmen at 0.05 obtained a P-value of 0.473 so that P-value > which means H0 is accepted. It can be concluded there is no relationship between the area of necrosis with the degree of lymphocytes infiltration in breast *Rattus norvegicus* induced by DMBA.

4. Discussion

Breast cancer is one type of malignancy that has a high prevalence which generally occurs in women, with a much higher prevalence.[8] The modeling of breast cancer in this study used the carcinogen 7,12 *dimethyl benzanthracene* (DMBA) with the consideration that this compound is the most potent polycyclic aromatic hydrocarbon group and can induce an inflammatory response to cause cancer induction.[9]

4.1 Degree of Lymphocyte Infiltration Area of Necrosis

We argue that the high percentage of cancer in the category of no area of necrosis can be caused by good immunosurveillance in mice so that it does not cause necrosis. Similar to Herberman study (2004) which states that mice that lose cellular immunity and are exposed to oncogenic agents will develop tumors more quickly.[10]

An overview of the area of necrosis in cancer model mice is shown in Figure 2 (C). The area of necrosis that occurs in experimental animals has a percentage of 41% of the total research sample. In this case, we argue that the emergence of areas of necrosis in cancer tissue can be caused by changes in the microenvironment such as Tumor Necrosis Factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), etc. that occur during the development of cancer tissue. The following study by Proskuryakov (2010) states that necrosis is considered a form of passive cell death in cancer tissue that cannot be reregulated.[11]

Another study stated that areas of necrosis in cancer tissue can be marked by a reddish color in the form of empty cells that can be found around abnormal cells in a cancer mouse model *(Sparageu dewly)*. Study-related to carcinogenesis by Alisah *et al* (2012) using DMBA induction also showed that DMBA was able to cause necrosis and the appearance of cancer cells in mice *(Sparageu dewly)*.[12,13]

4.2 Degree of Lymphocyte Infiltration

The results of the study in Table 2 show almost half have severe lymphocytic infiltration. The degree of lymphocyte infiltration in breast cancer tissue was found to vary even though it was induced with the same dose. In this study, it was also found that the degree of severe lymphocytic infiltration occurred more often than the degree of mild or moderate lymphocytic infiltration. We argue the high rate of lymphocyte infiltration is thought to occur due to an increase in strong antigenic stimulation from cancer cells to lymphocytes and causes an increase in lymphocyte proliferative activity.

Similar to Nakano's (2001) theory which showed that lymphocytes with lower proliferative activity may not perform sufficient effector functions due to the immunosuppressive environment in cancer tissue. Thus, variations in lymphocyte infiltration in cancer cells are very likely to occur depending on lymphocyte function.[14]

The results are shown in Figure 2 (D1, D2, D3) showed moderate lymphocyte infiltration in a mouse model of cancer-induced DMBA. Mild-moderate lymphocyte infiltration was found to be less than severe lymphocytic infiltration even though it was induced with the same dose. The degree of mild and

moderate lymphocyte infiltration is influenced by variations in the microenvironment in cancer tissue. Ekateriana study (2008) states that many factors affect the high or low infiltration of lymphocytes against cancer, one of which is the function of natural immune cells such as natural killer (NK) cells. Normally, cancer cells are recognized by NK cells and presented to lymphocytes via the Major Histocompatibility Complex (MHC-I). If this recognition process goes well, then the lymphocytes will respond in the form of releasing cytokines to proliferate the lymphocytes themselves. So that in some people can be seen to have a severe immune response.[15]

4.3 Relationship Area of Necrosis with Degree of Lymphocyte Infiltration

Statistical results in Table 3 show that there is no relationship between the area of necrosis and the degree of lymphocyte infiltration. We argue that the change of cancer cells into necrosis is not only caused by lymphocyte infiltration, but also due to other factors such as the degree of tissue ischemia due to anoxia (total oxygen supply inhibition) or cellular hypoxia (lack of oxygen to cells), as well as biological agents from bacterial toxins, viruses, and parasites.

Bacterial toxins can cause damage to blood vessel walls and thrombosis. Meanwhile, toxins derived from viruses and parasites can secrete various enzymes and toxins that directly or indirectly affect tissues. Chemical agents found in the body of mice such as sodium and glucose can disrupt the osmotic balance of cells which can affect the development of areas of necrosis. Physical agents can also affect the development of areas of necrosis due to physical trauma obtained by research mice during the study.[16]

In other studies, there is also evidence to support that the immune system has an important role in fighting cancer, especially breast cancer. Among them (a) Many tumors contain an infiltration of mononuclear cells consisting of CTL, NK cells, and macrophages; (b) Tumors may regress spontaneously; (c) Tumors develop more often in immunodeficient individuals or when immune system function is ineffective; even immunosuppression often precedes tumor growth; (d) On the other hand, tumors often cause immunosuppression in patients.[17,18]

5. Conclusion

More than a half of DMBA-induced *Rattus norvegicus* had no areas of necrosis. About a half of DMBA-induced *Rattus norvegicus* had severe lymphocytic infiltration. There is no relationship between the area of necrosis with the degree of lymphocytes infiltration in breast *Rattus norvegicus* induced by DMBA.

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