Effect of Papaya Leaf Extract on Cell Proliferation and Apoptosis Activities in Cervical Cancer Mice Model

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ABSTRACT

Cervical cancer is a primary cancer coming from cervix (cervical canal and or cervical portion). Tumor growth can occur due to uncontrolled proliferation and the reduced apoptosis. The development of anticancer treatment is directed at apoptosis induction. 

Carica papaya L. or papaya is one of the plants that are known for anticancer therapy by increasing the apoptosis and inhibiting the proliferation. Methanol extract of papaya leaves (Carica papain L) has inhibitory activity against DNA Topoisomerase II enzyme, an enzyme that has important roles in replication, transcription, and DNA recombination processes, and cancer cell proliferation will increase, and by the inhibition of this enzyme activity then there is a longer lasting bond between the enzyme and DNA and Protein-Linked DNA Breaks (PDBs) occurred and ended with apoptotic death. This research aimed at finding out that papaya leaves extract can act as anticancer, through the cell proliferation inhibition and apoptosis induction. This research uses true experiment method with 24 DDY mice aged 2 months, 20-30 grams from LPPT 4 (Integrated Research and Testing Laboratory) of UGM (GadjahMada University). Cervical cancer modeling was done by implanting HeLa cells in immunosuppressive mice with dexametazon injection of 0.5 mg/kg until 7 days. Furthermore, mice were injected at the back by HeLa cells of 1 ml intracutaneously (8x10^6 HeLa cell per mikrolit). Mice were observed for occurrence of nodule at injection area. Mice were divided into 4 groups: Control group (K), Treatment I (PI), Treatment II (PII), Treatment III (PIII). Each group consisted of six mice. After all groups were inoculated by cancer tumor cells and the nodule formed (1-2 weeks), then the mice were given the treatment as follows: K group with a standard diet without papaya leaf extract, P1 with a standard diet and papaya leaf extract of 225 mg/kg. P2 with a standard diet and papaya leaf extract of 450 mg/kg. P3 with a standard diet and papaya leaf extract of 750 mg/kg. Each group was given the treatment for 2 weeks. After the treatment period ended, mice were terminated by ether anesthetic and surgery for tumor tissue removal. Tumor tissues were processed into paraffin blocks, then preparation was made for histopathological examination by staining the tissues with HE (hematoxylin eosin). Then Ki67 immunohistochemical staining was performed to detect proliferation, and caspase-3 examination to detect apoptosis. The research results showed that proliferation of tumor tissues was detected using Ki-67 antibody with immunoratio analysis value of 54.7%, and apoptosis was detected using Caspase-3 antibody with immunoratio analysis value of 73.3%.

KEY WORDS: Cervical cancer, Proliferation, Apoptosis, Carica papaya L.

INTRODUCTION

Cancer is the second leading cause of death after heart disease, cancer deaths worldwide between 100 to 350 out of every 100.000 people annually [1,2]. Cervical cancer is a primary cancer coming from cervix (cervical canal and or portion) [2]. In Indonesia, the reported number of new cervical cancer was 100 per 100.000 population per year or 180.000 new cases between the ages in range of 45-54 years and ranks number one of 10 cancers most commonly found in women [3,4]. Tumor growth can occur due to uncontrolled proliferation and the reduced apoptosis. Cell proliferation is the cell division and cell growth. Impaired balance between apoptosis and proliferation is an important factor for the development and progression of tumor. Tumor growth can occur due to uncontrolled proliferation and the reduced apoptosis. Apoptosis and proliferation evaluation to know the growth or reduction of tumor mass [5,6,7].

Carica papaya L or papaya is one of the plants that are known for anticancer therapy by increasing the apoptosis and inhibiting the proliferation. Methanol extract of papaya leaves (Carica papain L) has inhibitory activity against DNA Topoisomerase II enzyme, an enzyme that has important roles in replication, transcription, and DNA recombination processes, and cancer cell proliferation will increase, and by the inhibition of this enzyme activity then there is a longer lasting bond between the enzyme and DNA and Protein-Linked DNA Breaks (PDBs) occurred and ended with apoptotic death. The objective of this research was to find out that papaya leaf extract can act as anticancer, through the inhibition of cell proliferation and apoptosis induction [8,9].

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MATERIALS AND METHODS

This research is a laboratory experimental research, using post test controlled group design with experimental animals of female mice. Research population includes C3H strain mice obtained from Integrated Research and Testing Laboratory (LPPT) of GadjahMada University in Yogyakarta. Research used 24 mice aged 2 months, 20-30 grams.

a. Preparation of Papaya Leaf Chloroform Fraction.

Test material of papaya leaves (Carica papaya L.) obtained from LIPI UPT Balaikonservasi Tumbuhan Kebun Raya Purwodadi (Center for Plant Conservation of Purwodadi Botanical Gardens, Indonesian Institute of Sciences) Pasuruan city, East Java, and extracted at the Nutrition Laboratory of Medicinal Raw materials in Airlangga University at the Faculty of Pharmacy.

To get papaya leaf chloroform fraction (Carica papaya L.), then 350 grams of papaya leaf powder (Carica papaya L.) was macerated beforehand with hexane solvent to remove the fat content (defatted). Maceration was done until the extract became clear. Dregs that have been macerated with hexane then re-macerated with acidic methanol (pH 3) added with 1% of tartaric acid (El-Sayyad, 1984), performed repeatedly until the extract becomes clear. The next step is to alkalimize the acid methanol extract with 5% of NH4OH until pH 9. This step is intended to hydrolyze the alkaloid in the form of salt to its base form so it can be pulled by organic solvent such as chloroform. The chloroform fraction was evaporated with a rotary evaporator to obtain chloroform fraction [8,9].

b. Preparation of experimental animals for cervical cancer

Cervical cancer modeling was done by implanting HeLa cell in immunosuppressive mice with dexametazon injection of 0.5 mg/kg until 7 days. Next, mice were injected in the back by HeLa cell of 1 ml intracutaneously (8x10^6 HeLa cell per mikrolit). Mice were observed for occurrence of nodule at injection area. Mice were divided into five groups: Negative Control group (K -), Positive Control group (K +), Treatment I (PI), Treatment II (PII), Treatment III (PIII). Each group consisted of 6 mice. After all groups were inoculated by cancer tumor cells and some nodules formed (1-2 weeks), then the mice were given the treatment as follows: K (-) with a standard diet without papaya leaf extract, K (+) with a standard diet and anti-cancer drug, P1 with a standard diet and papaya leaf extract of 225 mg/kg. P2 with a standard diet and papaya leaf extract of 450 mg/kg. P3 with a standard diet and papaya leaf extract of 750 mg/kg. Each group was given the treatment for 2 weeks.

After the treatment period ended, mice were terminated by ether anesthetic and surgery was performed for tumor tissue removal. Tumor tissues were processed into paraffin blocks, then preparation was made for histopathological examination by staining the tissue with HE (hematoxylin eosin). Then Ki67 immunohistochemical staining was done to detect proliferation, and caspase -3 examination was done to detect apoptosis.

c. Paraffin blocks preparation of tumor tissue.

Paraffin blocks containing cancer biopsy tissue were cut with a thickness of 4μm using a microtome, then deparafinated with xylol. Next, it was rehydrated with low-concentration ethanol, followed by Phosphate Buffered Saline (PBS) 3 x 5 minutes. Tissue preparation was incubated in Buffer DRAKOR Antigen Retrieval in the microwave with a temperature of 94° C for 20 minutes followed by cooling for 20 minutes at room temperature. Preparation was washed with PBS 3 x 5 minutes, and incubated at Block Peroxidase (Novocastra R). Next, the preparation was re-washed with PBS 3 x 5 minutes and incubated overnight with MIB-1 antibody at 4° C. After washing with PBS 3 x 5 minutes, the preparation was re-incubated with second antibody namely Novolink Horseradish Peroxidase (HRP), Novocastra R for 60 minutes at room temperature. After the incubation, the preparation was washed with PBS 3 x 5 minutes and counter stained with hematoxylin (Novocastra R). Next, preparation was dehydrated using high-concentration ethanol, followed by a purification process with xylol, and mounting [10,11].

d. Staining of Caspase-3 and Ki67 Immunohistochemistry

Microscopic preparation of tumor tissue was deparafinated with xylol, then rehydrated with low-concentration ethanol, followed by PBS 3 x 5 minutes. Tissue preparation was incubated in Buffer DRAKOR Antigen Retrieval in the microwave with a temperature of 98° C for 10 minutes followed by cooling for 20 minutes at room temperature. After washing with PBS/Tween 3 x 3 minutes, tissue preparation was incubated in Quench Peroxidase Cell Signaling R, and re-washed with PBS/ Tween 2 x 3 minutes. The next process is incubation in blocking solution for 60 minutes and primary antibody (apoptosis) at 4° C for one night. Preparation was washed with PBS/Tween 3 x 5 minutes then incubated in second antibody for 30 minutes and re-washed with PBS/Tween 3 x 5 minutes and DAB (DeaminoBenzidine) which is chromogenic for 2-10

minutes. Next, preparation was counterstained with hematoxylin eosin and dehydrated with high-concentration ethanol, purificated with xylol, and adhesion [11, 12].

Apoptosis staining was performed by immunohistochemical technique using Apoptosis Marker: Signal Stain Cleaved Caspase-3 (Asp175), IHC Detection Kit. While Ki67 staining also was performed with immunohistochemical technique using Rabbit Anti-Ki-67 (Proliferation Marker) Polyclonal Antibody – 100 UIBioss.

Apoptotic caspase-3 index which is the percentage of positive cancer tissue was evaluated randomly to avoid biased results. While Ki67 proliferation antigen identified as nuclear antigen was associated with cell proliferation. Cell cycle analysis in detail of Ki-67 has shown that the antigen contained in cell nucleus in all phases and mitosis phase, while in resting phase (G0) did not express Ki-67 [11,12].

Microscopic observation was performed by three randomly selected field of view, with at least 1000 cells (using images on 400x microscope magnification). To minimize variation, recount was performed in order to obtain a variation not more than 5%. Caspase-3 apoptosis index with level above 0.02 is classified as high apoptosis, and index level below 0.02 is rated as low apoptosis (18). Apoptosis index of microscopic images will be calculated based on their brown color by using online ImmunoRatiosoftware[11,12].

ETHICAL CLEARANCE
This research has passed the Ethical Clearance Test by Ethical Review Committee of the Faculty of Veterinary Medicine, Airlangga University by Number: 408-KE

RESULTS

1. Examination Results of HE (Hematoxylin and Eosin)
Histopathological examination results of mice skin organ with Hematoxylin and Eosin stainings showed that:

K (-) group: an inflammation area encapsulated by connective tissue was seen with neutrophil infiltration and macrophage in the center of inflammation. HeLa cells were seen in the center of inflammation.
K (+) group: Epidermal epithelial necrosis. There are hyperkeratosis, cell destruction, neutrophil infiltration, and lymphocyte in the epidermis.
P1 group: neutrophil infiltration, lymphocyte, and dermal and muscular plasma cells
P2 group: Epidermal epithelial necrosis. There are hyperkeratosis, cell destruction, neutrophil infiltration, and lymphocyte in the epidermis.
P3 group: There are no pathological changes, skin structure looks normal.

Figure 1. Histopathological results of mouse skin organ with hematoxylin and eosin (HE)staining

K (-) group: an inflammation area encapsulated by connective tissue was seen with neutrophil infiltration and macrophage in the center of inflammation. HeLa cells were seen in the center of inflammation.
K (+) group: Epidermal epithelial necrosis. There are hyperkeratosis, cell destruction, neutrophil infiltration, and lymphocyte in the epidermis.
P1 group: neutrophil infiltration, lymphocyte, and dermal and muscular plasma cells
P2 group: Epidermal epithelial necrosis. There are hyperkeratosis, cell destruction, neutrophil infiltration, and lymphocyte in the epidermis.
P3 group: There are no pathological changes, skin structure looks normal.
2. Apoptosis Index Identification with Caspase-3 Antibody

Figure 2: Immunohistochemical Examination Results of cancer cell apoptosis by caspase-3 antibody for Negative Control group treatment of 7.6% (Figure A), and positive control treatment with apoptosis index of 8.5% (Figure B), papaya leaf extract treatment at a dose of 500 mg/kg with apoptosis index of 73.3% (Figure C) indicated by brownish color with ImmunoRasio analysis.

3. Proliferation Activity Identification with KI-67 Antibody

Figure 3: Immunohistochemical Examination Results of cancer cell proliferation by KI-67 antibody for negative control group with proliferation activity of 57.4% (Figure A), and positive control group with proliferation activity of 24.4% (Figure B) and papaya leaf extract treatment group at a dose of 500 mg/kg with proliferation activity of 8.0% (Figure C) indicated by brownish color with ImmunoRasio analysis.

DISCUSSION

a. Identification of apoptosis expression of cancer cell with Caspase 3 antibody

Observation results on microscopic preparation of cervical cancer tissue by immunohistochemical examination on apoptosis expression using caspase 3 antibody are shown in Figure 2. Apoptosis expression of caspase-3 (brown) in cervical cancer cell nucleus of caspase-3 can be seen in Figure 1-3. Apoptosis evaluation by immunohistochemical examination with caspase 3 antibody showed the average value of 73%.

This research used papaya leaf extract at 3 dose i.e. 225, 450, 750 mg/kg. Of the three doses used, the second dose of 450 mg/kg provides the greatest apoptosis expression indicated by caspase-3 expression value of
Ki-67 is one of cell proliferation markers that is useful to determine growth fraction in tumor cell during active phase of cell cycle [16,22]. Ki-67 expression is evaluated by three groups: low grade (<15%), intermediate (16-30%), and high grade (>30%) [15,21].

**b. Identification of inhibition expression of cancer cell proliferation by Ki67 antibody**

Observation results on microscopic preparation of cervical cancer tissue by immunohistochemical examination on proliferation expression using Ki67 are shown in Figure 2. Proliferation expression of Ki67 (brown) in cervical cancer cell nucleus can be seen in Figure 2B. Proliferation evaluation by immunohistochemical examination with Ki67 antibody showed the average value of 54.7%.

Ki-67 proliferation antigen identified as nuclear antigen is associated with cell proliferation. Cell cycle analysis in detail of Ki-67 has shown that the antigen existed in cell nucleus in all phases and in mitosis phase, while in resting phase (G0) did not express Ki-67. Although the structure and property of this protein has been understood, but its functional role is still not known exactly [15].

Ki-67, BRCA, and p53 genes are linked when they are proto-oncogene, and tumor suppressor gene that normally functions to control cell growth, but if a mutation happens in this three genes then the cells are uncontrolled and hyperproliferation may occur. P53 is a tumor suppressor gene and effector molecule checkpoint of G1, G2, S, and M phases through induction of cell-cycle inhibitor p21 causing cessation of cell cycle. Mutation in p53 gene will cause uncontrolled cell proliferation. BRCA is also a tumor suppressor gene that has a critical role in repairing the broken double-strand DNA in which the reduced BRCA due to mutation may result in gene instability and cause tumor cell growth [16].

Ki-67 is a non-histone nuclear protein which has two isoforms with a molecular weight of 359kD and 320kD, this gene is located on chromosome 11q25, this protein is found in nucleolus cortex and dense fibrin component in nucleolus during interphase phase. The half-life of Ki-67 ranged between 1-1.5 hours. Healthy breast tissues express Ki-67 in a low level (<3%) [17,18].

Ki-67 expression varies according to cell cycle phase. Cells express this antigen during Growth phase-1 (G1), Synthetic phase (S), Growth phase-2 (G2) and Mitotic phase (M), but not express them at the resting phase (G0). Ki-67 level is low in G1 and S phases, it begins to rise until it reaches its highest point at mitotic phase whereas the expression decreases sharply at anaphase and telophase. This protein plays an important role in cell division process, but until now the definite function of this antigen is not yet known [15].

Ki-67 is a biological marker that can reflect the state of cell proliferation. Many data show that Ki-67 is a prognostic factor in carcinoma mammae [15]. Ki-67 is a protein in tissues that experience division and show that this protein plays an important role as a cell division marker. Increased level will be followed by a lesion with a high grading and microinvasion, therefore, it is not surprising that Ki-67 is a predictor of recurrence in ductal carcinoma in situ (DCIS) [19].

Cell proliferation is the division and growth of cell, whose mechanism and regulation are based on the existence of cell cycle. Cell proliferation activity can be detected using Ki-67 immunohistochemical staining. Ki-67 will be expressed in proliferating cell in Growth phase-1 (G1), Synthetic phase (S), Growth phase-2 (G2), Mitotic phase (M) except the resting phase (G0) of cell cycle [15].

Cell normally experiences mitotic division in a cycle called the cell cycle, serves to generate new cells that are useful for regeneration and to repair the damage, this cycle is governed by DNA sequence in each cell. Cell has gene that regulates cell proliferation called a protooncogene, such as Ki-67 gene and genes that function to regulate termination or inhibition of cell proliferation called gene suppressor such as p-53. These genes serve as control, if these genes are mutated, then the related protein does not form properly and a cell division may happen unnecessarily. Ki-67 gene mutation in mitosis phase may cause uncontrolled cell division resulting in cell movement that experiences the division and stimulates tumor cells to go through damaged basal membrane or lysis and go into the circulation (blood flow). Tumor cell that forms this lump will spread haematogenously and eventually come into the blood vessel and can invade directly the blood vessel through the vena cava so that it can be detected in the blood [20].

Ki-67 is one of cell proliferation markers that is useful to determine growth fraction in tumor cell during active phase of cell cycle [16,22]. Ki-67 expression is evaluated by three groups: low grade <15%, intermediate 16-30%, and high grade > 30%) [15,21].
CONCLUSION

Papaya leaf extract provides the greatest apoptosis expression indicated by caspase-3 expression value of 73.3%, and inhibition expression of cancer cell proliferation by KI67 antibody showed the average value of 54.7%.

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REFERENCES

19. Annals of Oncology. Is the Ki-67 Labelling Index ready for Clinical Use? Published by Oxford University Press on behalf of the European Society for Medical Oncology, 2011.
21. Hassan. Ki-67 marker useful for classification of malignant invasive ductal breast cancer. Department of Anatomical Pathology, Faculty of Medicine, 2013