The Study on Apoptosis of The Liver Cells White Rats Due to the Different Exposure Times and Dosages of Aflatoxin B_1

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Abstract

Apoptosis is known to maintenance of tissue homeostasis and elimination of cancer cells. Whithout programed cell death, cell proliferation would lack on important component of control of oncogenic process. Apoptosis is caused by various inducers such as chemival compound and toxin. Aflatoxin B_1 is a potent toxin. In the liver, it undergoes biotransformation which produces reactive oxigen species, causes cellular stress that initiates apoptosis. However, the correlation between exposure of AFB and the evidence of apoptosis in the liver and development of Hepato Cell Carcinoma has not been elucidated.
For this purpose, we used an animal experiment with 96 white rats (Rattus Norvegicus). Adult healthy white rats were divided into four groups of 24 rats each, based on the dosages of AFB_1 given. Each group was divided further into three subgroups of eight rats based on the length of exposure time to AFB_1 .
Four dosages of AFB_1 , were introduced orally everyday into different groups, consisted of 0, 10, 15 and 20 µg dissolved in 0,2 ml propylene glycol. Three subgroups received the dosage for 12 weeks, 16 weeks, and 20 weeks. At the end of the experiment, the rats were sacrificed. Liver cells with apoptosis were scrutinized using peroxidase insitu apoptosis detection kit and liver cell damages were examined using histological slices stained by haematoxillin cosin.
In our analysis, we found that apoptosis of the liver increased until the formation of dysplacia of the liver cells. After that apoptosis decreased. It means that the highest dosages and the longest time exposure AFB_1 inhibited apoptosis.
We concluded that apoptosis of the liver cells due to AFB_1 caused not only by the damaged of mitochondria (caused by reactive oxygen species) but also by the mutation of p53 which we could see dysplacia of the liver cell in histogical slices.

Key word : apoptosis, aflatoxin B_1, dysplacia, radical oxygen species

Introduction

Apoptosis, programmed cell death, is known to participate in various biological processes such as development, maintenance of tissue homeostasis and elimination of cancer cells (Bossy and Green., 1995; Norburg and Hickson, 2001; Cotran *et al*., 1999). Malfunction of apoptosis have been implicated in many froms of human diseases such as neurodegenerative diseases, AIDS, and ischemic stroke. Without programmed cell death, cell proliferation would lack an important component of control of oncogenic processes. Reportedly, apoptosis is caused by various inducers such as chemical compounds and toxins (Kuwana *et al*., 1998; Foster and Rosche, 2001).

Aflatoxins are a group of toxic metabolites produced by the mould Aspergillus flavus. Among this toxins, Aplatoxin B1 is the most potent naturally occurring carcinogens and is classified as group I carcinogens by international Agency for research on Cancer (IARC) (Narasimhan *et al* 2000). Warm temperature, high humidity and plant injuries, in the field and during storage, promote both the growth of the fungi and aflatoxin production (Foster and Rosche, 2001). these toxins are encountered in certain areas endemic for hepatocelluler carcinoma (Cotran *et al*., 1999).

The mechanism of action AFB_1 on the cell is mediated through the production of free radicals of reactive oxygen species (ROS) (Narasimhan *et al* 2000). It has been proven that AFB_1 induced lipid peroxidation on the rat liver and decreased antioxidant defense in the body (Narasimhan *et al* 2000; Shen *et al*; 1994; Yanwirasti, 2004). The situation of a serious imbalance between production of ROS and antioxidant defense can result oxidative stress (Shen *et al*; 1995).

Oxidative stress can damage to all type of biomelecule (including DNA, proteins and lipids), dysregulation Ca^{2+} metabolism, and damage of mitochondria. Rises in Ca^{2+} - dependent endonucleases in the cell nucleus to cause DNA fragmentation, an event which is important in apoptosis.

The aim of this study was to disclose apoptosis on the liver cell white rats produced by different dosages and exposure times of Aflatoxin B1.

Materials and Methods

1. Chemical and reaction kit
   AFB1 was purchased from sigma grade A – 6636 Saint Louis, USA. Detection of apoptosis by using, Apoptag peroxidase Insitu, (Apoptosis detection kit) was purchased from Intergen company.
2. Animals and experimental design
   Ninety six male white rats (Rattus norvegicus) were purchased from Animal center of Gajah Mada University (Jogjakarta). They were divided into four groups of 24 rats each, based on the dosages of aflatoxin B1 given. Each group was divided further into three subgroups of eight rats based on the length of the exposure time to AFB1. Four dosages of AF B1 were administered orally every day into different groups, consisted of 0 µg, 10 µg, 15 µg, and 20 µg, dissolved in 0,2 ml propylene glycol. Three groups received the dosage for 12 weeks, and 20 weeks. At the end of the experiment, the rats were sacrificed, and the liver fixed in 4% neutral formalin. Detection of apoptosis in the liver cells were scrutinized using in situ detection of apoptotic cells in paraffin – embedded tissue and subsequent visualization by light microscope. We also examined liver cell damages using histological slices stained by haematoxillin eosin.

Results

To explore apoptosis in the liver cells produced by the different dosages and time, we examined by using in situ detection of apoptosis cells in paraffin – embedded tissue. As shown in table 1, apoptosis liver cells increased until dosage 10 µg and duration 16 weeks, but after that there was no increasing apoptosis on the liver cells for adding dosages and length of induction of AFB1.

Table 1. Apoptosis on the liver cells based on the length and dosage of AFB1 given

The length of time AFB1 given	The dosages of AFB1 given (µg)			
	0	10	15	20
	Mean	mean	mean	Mean
12 weeks	0.50 ± 0.54	5.38 ± 1.77	6.38 ± 2.13	7.75 ± 2.25
16 weeks	0.38 ± 0.74	10.50 ± 2.67	9.13 ± 2.74	9.13 ± 2.36
20 weeks	0.25 ± 0.46	8.0 ± 1.77	7.38 ± 2.10	7.75 ± 2.05

Table 2. Tukey HSD apoptosis on the liver cells based on dosages, length, and interaction of AFB1 given

Dosage (µg)	Dosage (µg)	0			10			15			20		
	Length Of time (weeks)	12	16	20	12	16	20	12	16	20	12	16	20
0	12	-	ns	ns	s	s	s	s	s	s	s	s	S
	16		-	ns	s	s	s	s	s	s	s	s	S
	20			-	s	s	s	s	s	s	s	s	S
10	12				-	s	ns	ns	s	ns	ns	s	ns
	16					-	s	ns	ns	ns	ns	ns	ns
	20						-	ns	ns	ns	ns	ns	ns
15	12							-	ns	ns	ns	ns	ns
	16								-	ns	ns	ns	ns
	20									-	ns	ns	ns
20	12										-	ns	ns
	16											-	ns
	20												-

Ns – non significant ($p > 0.05$) s = significant ($p < 0.05$)

To analyze apoptosis on the liver cells based on dosages, length, and interaction of dosage and length of AFB1 given, we used Tukey HSD as shown in table 2

Table 3. Dysplacia on liver cells based on dosages and length of time AFB1 given / 50 cells

The length of time AFB1 given	The dosages of AFB1 given (µg)			
	0	10	15	20
	Mean	Mean	Mean	Mean
12 weeks	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
16 weeks	0 ± 0.00	0 ± 0.00	3.38 ± 1.84	5.62 ± 3.16
20 weeks	0 ± 0.00	0.88 ± 0.84	5.88 ± 4.36	8.63 ± 6.94

Tukey HSD showed that apoptosis on the liver had significantly higher between group length 12 weeks, 16 weeks, and 20 weeks with dosages 10 µg, 15 µg, and 20 µg of AFB1 given compared with groups length 12 weeks, 16 weeks and 20 weeks with dosage 10 µg of AFB1 given. No significant difference on apoptosis of the liver cells was found between the length 12 weeks, 16 weeks, and 20 weeks with dosages 10 µg, 15 µg, and 20 µg of AFB1 given.

From the table 3, dysplacia on the liver cells occurred at the dosage 15 µg and 16 weeks of AFB1 given. The more increased of dosage and time of AFBi given, the more dysplacia on the liver cells occur. To analyze dysplacia on the liver cells based on dosages, length and interaction of AFB1 given, we used tuket HSD

Table 4. The significance of HSD test of dysplacia on the liver cells based on interaction dosages and length of times of AFB1 given.

Dosage (µg)	Dosage (µg)	0			10			15			20		
	Length Of time (weeks)	12	16	20	12	16	20	12	16	20	12	16	20
0	12	-	ns	ns	ns	ns	Ns	ns	ns	s	ns	s	s
	16		-	ns	ns	ns	ns	ns	ns	s	ns	s	s
	20			-	ns	ns	ns	s	ns	s	ns	s	s
10	12				-	ns	ns	ns	ns	s	ns	s	s
	16					-	ns	ns	ns	s	ns	s	s
	20						-	ns	ns	s	ns	s	s
15	12							-	ns	s	ns	s	s
	16								-	ns	ns	ns	s
	20									-	s	ns	ns
20	12										-	s	s
	16											-	ns
	20												-

Ns – non significant ($p > 0.05$) s = significant ($p < 0.05$)

From the table 4, we found that there was significant differences between the effect of 20 µg dosage and the length of 16 weeks and 20 weeks of AFB1 given with 10 µg and 12, 16, and 20 weeks of AFB1 given, dosage 20 µg and 12 weeks of AFB1 given.

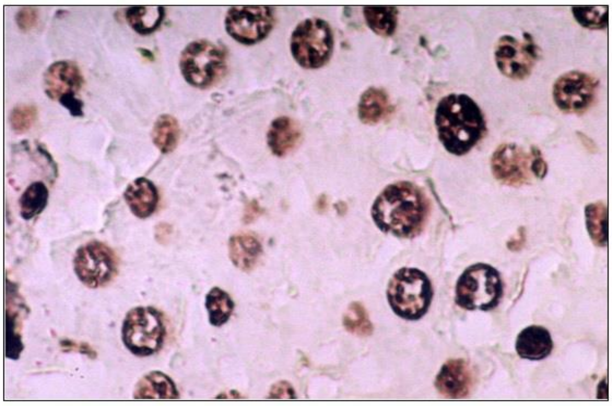

Fig. 1. Apoptosis on Hepatocyt cells after AFB1 given. (400x)

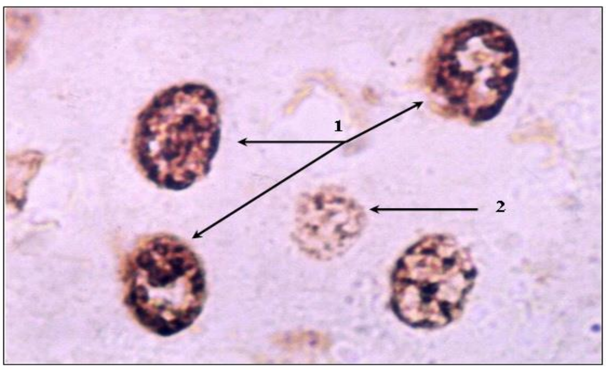

Fig. 2. Apoptosis on Hepatocyt cells after APB1 Given (1000x)

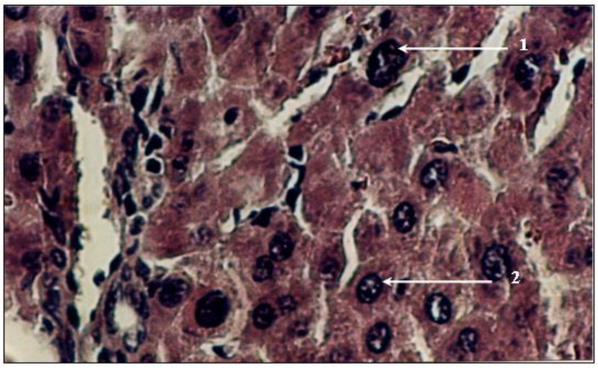

Fig. 3. Dysplacia on Hepatocyt (He, 400x)

Discussion

Bioactivities of aflatoxins has been demonstrated as a necessary step for cytotoxic and genotoxic effects (Bossy and Green, 1995). Cytocrome P450s (Cy P450s) are the principal enzymes involved in the oxidative biotransforamtion of AFB1 (Mace *et al*., 1997). The P450 reaction cycle can also generate superoxide and H2O2. The reaction of superoxide and H2O2 produced OH^0, a kind of reactive oxygen species (Preddy *et al*., 1998). Increased production of ROS, which caused by presence of toxin AFB1, diminished antioxidant in the body. Imbalanced of antioxidant and ROS produced oxidative stress caused oxidative damage (Halliwell and Gutteridge, 2004). It has been proven in our previous study, that AFB1 decreased enzymes SOD and catalase of liver tissue and increased malondialdehyde of liver tissue and liver cell damage (Yanwirasti, 2004). Under the increased of ROS which produced by bioactivities AFB1, the permeability of the inner mitochondrial membrane was lost because of opening of the pores in the membrane that allowed movement of molecules. Normally, the membrane is impermeable. Pore opening resulted in rising intracellular Ca^{2+} levels. Rises in Ca^{2+} can also activated Ca^{2+} - dependent endoucleasis in the cell nucleus to cause DNA fragmentation, an event which was important in apoptosis (Cotran *et al.*, 2004).

ROS which produced by bioactivities AFB1, triggered mitochondria to release caspase – activating protein, among which were cytochrome C. Cytochrome C activated caspases by binding to Apaf – 1, inducing it to associate with procaspase 9, there by triggering caspase – 9 activation and iniating the proteolytic cassade that culminates in apoptosis (Green and Real, 1998; Mann and Cidlowski, 1999; Lee *et al.*, 2001).

In our study, we found that apoptosis on the liver cells increased until dosage 10 µg and 16 weeks of AFB1 given. There were only significant differences of apoptosis on the liver cells between dosage 10 µg and 16 weeks of AFB1 given with dosage 10 µg and 12 weeks of AFB1 given ($p < 0.05$) and dosage 20 µg and 12 weeks of AFB1 given. But, there were no increasing apoptosis on the liver cells anymore by increasing dosages and length of time AFB1 given. The analyzed of data, we found that there were no significant differences of apoptosis on the liver cells between dosage 10 µg and 16 weeks of AFB1 given with dosage 10 µg and 20 weeks AFB1 given ($p > 0.05$), dosage 15 µg and 16 weeks and 20 weeks AFB1 given ($p > 0.05$), dosage 20 µg and 12 weeks, 16 weeks and 20 weeks AFB1 given ($p > 0.05$). It means that apoptosis not only caused by the damage of mitochondria, but also by the damage of DNA, which involved the tumor suppressor gene p 53.

Oxidative stress caused by exposure AFB1, can damage DNA, Wt – P53 protein function as a transcription factor, positively regulating a number of genes by interacting with specific DNA regions (Bates, 1996). Exposure of ROS which produced by bioactivities AFB1 led to a noticeable rise of p 53 protein. It has been demonstrated by Yang and Hughes in 1998, that after exposure 20 µg/ml AFB^1 for 16 h on NCTC 929 mouse fibroblast cells, induction of p 53 was apparent which was analyzed by both Eisa and Western Bot (Yang and Hughes, 1998). Rising of p 53 protein required for DNA repair or apoptosis. Manipulations that cause an increase in p 53 expression have been shown to result in apoptosis, for example, following the introduction of cloned p 53 genes into some tumor cells lines. Additionally, rising p 53 levels in normal mouse thymocytes by exposing then to agent that damage DNA leads to apoptosis (Oliner, 1994). The most increase induction of AFB1, the most damage of DNA. It made lacking functional p 53 as growth – suppressive role, resulting in inefficient DNA repair and the emergence of genetically unstable cells and inactivate p 53 – mediated apoptosis (Soussi *et al*., 2000).

AFB1 is known to be a major risk factor for the development of hepatocellular carcinomas (HCC) in many areas of the world (Wang *et al*. , 1999). It has been postulated to be a hepatocarcinogen in human by causing p 53 mutation (Mc Glynn *et al*, 1995; Smella *et al*, 2001). Ag to T transversion at third position of codon 249 of the p 53 gene is commonly found in HCC from patients in regions with dietary aflatoxin exposure. In vitro studies have supported this finding, showing that AFB1 can induced this mutation (Stern *et al*., 2001). An animals experiment had shown that p 53 point mutation enhanced by hepatic regulation in AFB1 – induced rat liver tumor and pre neoplastic lesion.

In our study, we found that apoptosis decreased at dosage 15 µg and 16 weeks AFB1 given. At the same time, we found that there were dysplacia on liver cells by using haematoxillin eosin stained slices. Dysplacia was an abnormal organization of cells, in addiction to proliferating excessively, the off spring of this cell appear abnormal in shape and in orientation. Dysplacia began when some cells within a normal population sustained a genetic mutation (Weinbeg, 1996). In our study, dysplacia began at the lack functional p 53 as activate p 53 – mediated apoptosis. The loss of the function of p 53 was important step in carcinogenesis because the mutation of p 53 disrupt apoptosis and cause tumor initiation (Lee *et al*., 1998). Table 3 showed that the higher of AFB1 induction, the higher dysplacia we had. It was difference with apoptosis, whereas the higher of AFB1 induction the lower apoptosis we had.

We concluded that the mechanism of apoptosis on the liver cells induced by AFB1 happened by two ways: first, role of mitochondria and second, role of p 53 gene.

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