한국수의병리학회지. Korean J Vet Pathol 1 : 33~39(1997)

Pathological Changes in Rats Fed *Petasites japonicus* Maxim II. Immunohistochemical Localization of Cytochrome P4502E1 and GST-P in Liver

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Abstract. We investigated metabolism and carcinogenesis in livers of Sprague-Dawley rats fed juices and pelleted diets containing Korean native plants, *Petasites japonicus* Maxim, by evaluating cell localization and expression of cytochrome P450s and GST-P. Anti-cytochrome P450s application in liver sections revealed three to four times increased expression of cytochrome P4502E1 immunoreactivity in degenerative hepatocytes when compared to histologically normal hepatocytes. Anti-GST-P in showed positive preneoplastic foci as well as in individual hepatocytes randomly scattered throughout all liver sections examined. Additionally, GST-P was evident in proliferative endothelial cells and biliary epithelial cells in exposed rat livers. These results suggested that the increased level of cytochrome P4502E1 in affected hepatocytes was a direct consequence of *Petasites japonicus* toxicity. Further, immunoreactivity to anti-GST-P in hepatocytes, endothelial cells and biliary epithelial cells indicated a possible preneoplastic effects of *Petasites japonicus* in Sprague-Dawley rat.

Key words: Petasites japonicus Maxim; cytochrome P4502E1; glutathione S- transferase placental form(GST-P); preneoplastic lesion; endothelial cell and biliary epithelial tumor.

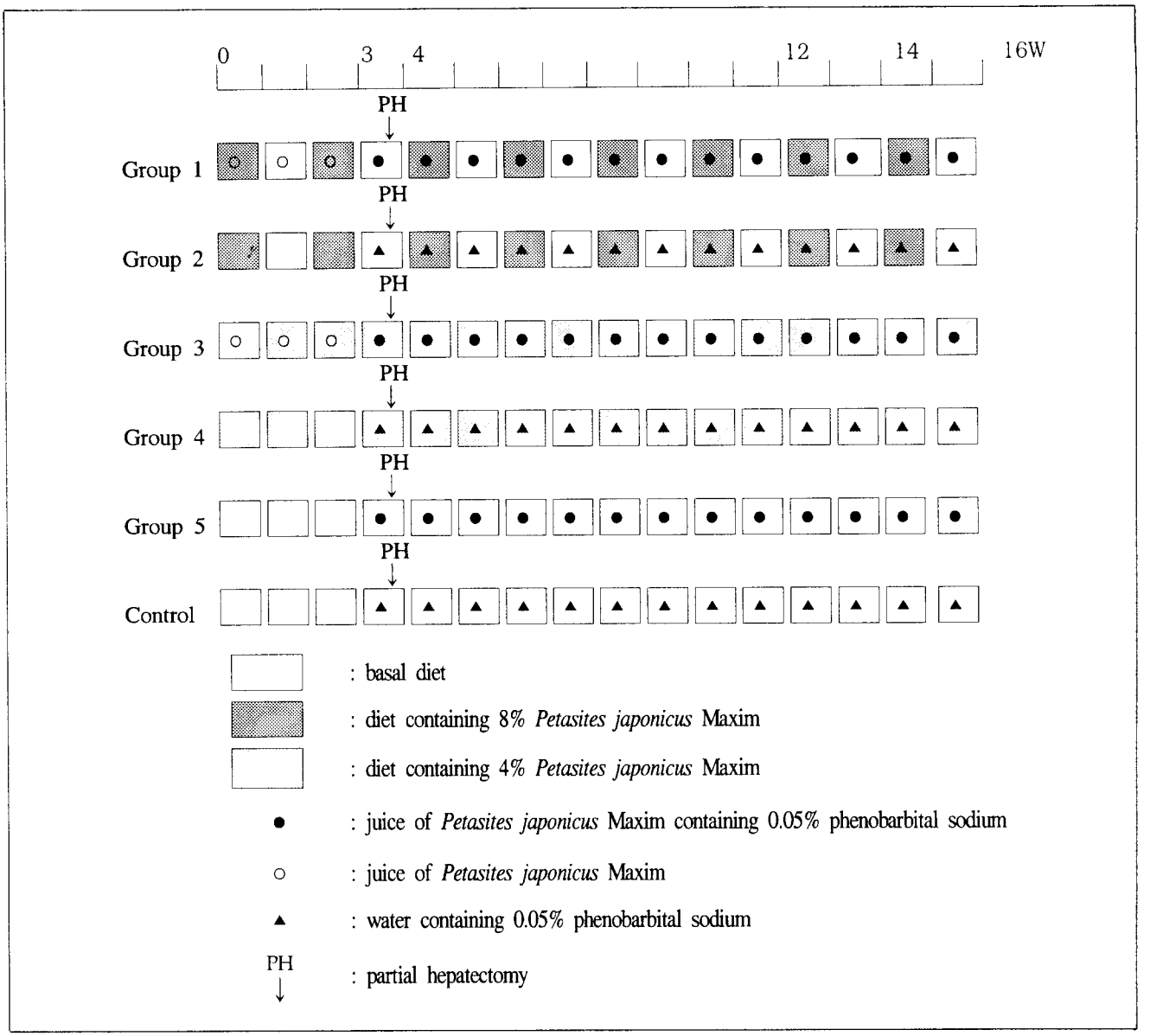
Hepatocarcinogesis has been reported to be caused by viruses, mycotoxins, various chemicals, drugs, environmental factors, and plants[2]. The liver represents the major target for many carcinogenic factors, because it is continuously exposed to endogenous- and exogenous factors. Many of these xenobiotics require bioactivation to exert cytotoxic or tumorigenic effects[13].

Representative naturally occurring carcinogenic plants are the tobacco plant, and the bracken fern, which have been used to study their carcinogenic activity in different species of animals[28]. The best known of all plant carcinogens with a major impact as a cause of human cancer is the group of agents in the tobacco plant[45]. It is known that the smoke contains many procarcinogens(e.g.) polycylic aromatic hydrocarbons, nitrosamines, and aromatic(amines) that induce deleterious effects after activation. A derivative of safrole, dihydrosafrole, yielded tumors in the esophagus of rats, through unknown mechanisms. Bracken fern caused urinary bladder cancer which seems to be the main lesion in several species[22]. Fiddleheads of bracken fern are used as human food and therefore are of public health concern.

We have reported congestion, hemorrhage, fatty change, focal necrosis, megalocytosis, and proliferative endothelial cells and biliary epithelial cells in livers of rat fed juices or pelleted diets containing the Korean native plant *Petasites japonicus* that grows along the roadsides and the waterways of flatlands, in the moutainous districts, and around houses[21]. Previous studies[10] also have reported the possible carcinogenicity as well as toxic effects of *Petasites japonicus,* histopathologically. It is fairly clear that primary tumors in the liver are caused by administration of *Petasites japonicus* for long periods[17,18,19].

Hepatocarcinogenesis is a multistep process involving successive stages of initiation, promotion, and progression of lesions from preneoplastic foci to benign and malignant neoplasms[26]. Recently, it has been applied to the mid-term bioassay[20,31] and short-term assay system[3,44] in order to keep and expense to a minimum. Following limited exposure to carcinogens, enzyme altered foci which are regarded as comprising preneoplastic hepatocytes, appear in the liver[19]. As for phenotypic expressions, and the activities of enzyme markers, negative ones are glucose-6-phosphatase, adenyl cyclase, adenosine triphosphatase, glycogen phosphorylase, tryptopan oxygenase, and alkaline nuclease, while positive ones are glycogen, glucose-6-phosphate dehydrogenase, microsomal epoxide hydrolase, UDP-glucuronyl transferase, γ -glutamyl transpeptidase(γ -GT)[4,15,31,32], and glutathione S-transferase placental form(GST-P)[13,34].

Among various enzyme-histochemical markers characterizing preneoplastic liver lesions in rodents, γ -GT has been widely used. More recently, the placental form



Text-Fig. 1. Schematic representation of experimental regimens in rats

of GST-P has been recommended as a suitable immunohistochemical marker[30]. Both γ-GT and GST-P are considered to be phase II enzymes (detoxification of xenobiotics) and are related to interactions of glutathione during hepatocarcinogenesis. The GST-P, so called GST 7-7 substrate specificity is generally broader, and binding ability for diverse organic compounds such as bilirubin, hematin, and sulfobromophthalein was as high as in any of the other six forms[35,38,39] In contrast to γ-GT histochemistry, GST-P immunohisto- chemistry does not give rise to an apparent positive staining of normal hepatocytes, though some anti-oxidant drugs that are strong γ-GT inducers are reported to induce the enzyme slightly in non-preneoplastic hepatocytes[30,33,37,38,41]

Among xenobiotic metabolizing enzymes, the cytochrome P450s dependent mono-oxgenase system plays a major role in the metabolism of numerous important groups of chemicals, including drugs, carcinogens, steroids, pesticides, hydrocarbons, and natural products[13]. The toxicity of these compounds may be modulated, for better or for worse, by the various oxidating steps catalyzed by the cytochrome P450 enzymes[13]. Among various isozymes of P450s, cytochrome P4502E1 has been induced by administration of ethanol and this isozyme has been characterized and identified as a polypeptide with minimal Mr 51,000~55,000 in animals, respectively[34]. The substrates for P4502E1 include ethanol, propanol, acetone, ether, benzene, carbon tetrachloride, and certain carcinogens[13]. The active metabolites are often electrophilic compounds which can bind to cellular proteins and nucleic

Table 1. Time-dependent expression of GST-P positive proliferative endothelial and billary epithelial cells of rats fed Petasites japonicus Maxim.

Time after administration (weeks)	Age (weeks)	Incidence of GST-P immunoreactivity					
		Group					
		1	2	3	4	5	6
12	3	2/5	0/1	1/3	0/1	1/2	0/2
		0/1	0/1	1/1	0/2	0/1	0/1
14	5	1/2	2/3	3/4	2/4	1/3	0/4
		1/2	0/1	0/1	0/3	1/2	0/2
16	3	2/2	2/2	1/2	1/3	2/3	0/4
		3/4	2/3	2/2	2/4	2/2	0/4
Total	5	5/8	4/6	5/9	3/8	4/8	0/10
		4/7	2/5	3/4	2/9	3/5	0/7

Group 1 ; Juice and diet containing 8% dried *Petasites japonicus* Maxim. for 1 week and a normal diet for 1 week alternatively.
Group 2 ; Diet containing 8% dried *Petasites japonicus* Maxim for 1 week and a normal diet for 1 week alternatively.
Group 3 ; Juice and diet containing 4% dried *Petasites japonicus* Maxim.
Group 4 ; Diet containing 4% dried *Petasites japonicus* Maxim.
Group 5 ; Only juice.
Group 6 ; Control

acids and interfere with cell function, resulting in cell toxicity[12]. Most of these studies have been performed using liver as the target, and little information is available on the isozyme types of cytochrome P4502E1 in extra hepatic organs or tissues[43]. The cellular localization of basal- and induced cytochrome P4502E1 is also an important determinant of toxicity[13].

For example, helenalin, a natural plant product with significant anti-tumor activities, was reported by decreased male BDF1 mouse hepatic microsomal cytochrome P450 levels *in vivo and in vitro*[9].

There has been no report on the metabolism of petasitenine from the leaves and stalks of wild Korean native *Petasites japonicus*, which has been wdely used as human food. In the present study, we have investigated enzyme induction in rats fed juices and pelleted diets containing *Petasites japonicus* by immunohistochemical study of cytochrome P450s and GST-P.

Materials and Methods

Animals and diet preparation

Sprague-Dawley rats(3 weeks old, 5 weeks old) were obtained from Korea Atomic Energy Rearch Institute(Seoul, Korea). The animals were housed in polycarbonate cages according to groups. *Petasites japonicus* was collected in Kyungpook local areas in May through June. The rats were fed with juices and pelleted diets(4% or 8%) of leaves and stalks of wild *Petasites japonicus* and water ad libitum. To prepare the pellets, the fresh Petasites japonicus was dried, milled, and then mixed with the rat basal diet(Samyang Co., Korea).

Experimental designs

Rats were divided into 6 groups, each consisting of 10 ~ 17 animals. They were divided into 5 groups fed *Petasites japonicus* or the juice and 1 group as a control. After 4 weeks, in all the groups under ether anesthesia, portions of the hepatic parenchyma, ranging in extent from 65% to 75% of the total liver, were removed, leaving within the peritoneum the right lateral lobe and the small caudate lobe[26]. All animals were sacrificed first at 12, 14 weeks, and 16 weeks, respectively(Text-Fig. 1).

Group 1: Three weeks old(n=9) and five weeks old(n=7) rats were fed juice and 8% pelleted diet of *Petasites japonicus* for 1 week and a normal diet for 1 week alternately. Three weeks later, they were provided with juice containing 0.05% phenobarbital sodium(PB)(Daewon pharm. Co., Korea).

Group 2: Three weeks old(n=6) and five weeks old(n=5) rats were fed 8% pelleted diet of *Petasites japonicus* for 1 week and a normal diet for 1 week alternately. Three weeks later, they were provided with water containing 0.05% PB.

Group 3: Three weeks old(n=9) and five weeks old(n=4) rats were fed juice and 4% pelleted diet of *Petasites japonicus*. Three weeks later, they were provided with juice containing 0.05% PB.

Group 4: Three weeks old(n=8) and five weeks old(n=9) rats were fed with 4% pelleted diet of *Petasites japonicus*. Three weeks later, they were provided with water containing 0.05% PB.

Group 5: Three weeks old(n=8) and five weeks old(n=7) rats were fed a normal diet and juice of *Petasites japonicus*. Three weeks later, they were provided with water containing 0.05% PB.

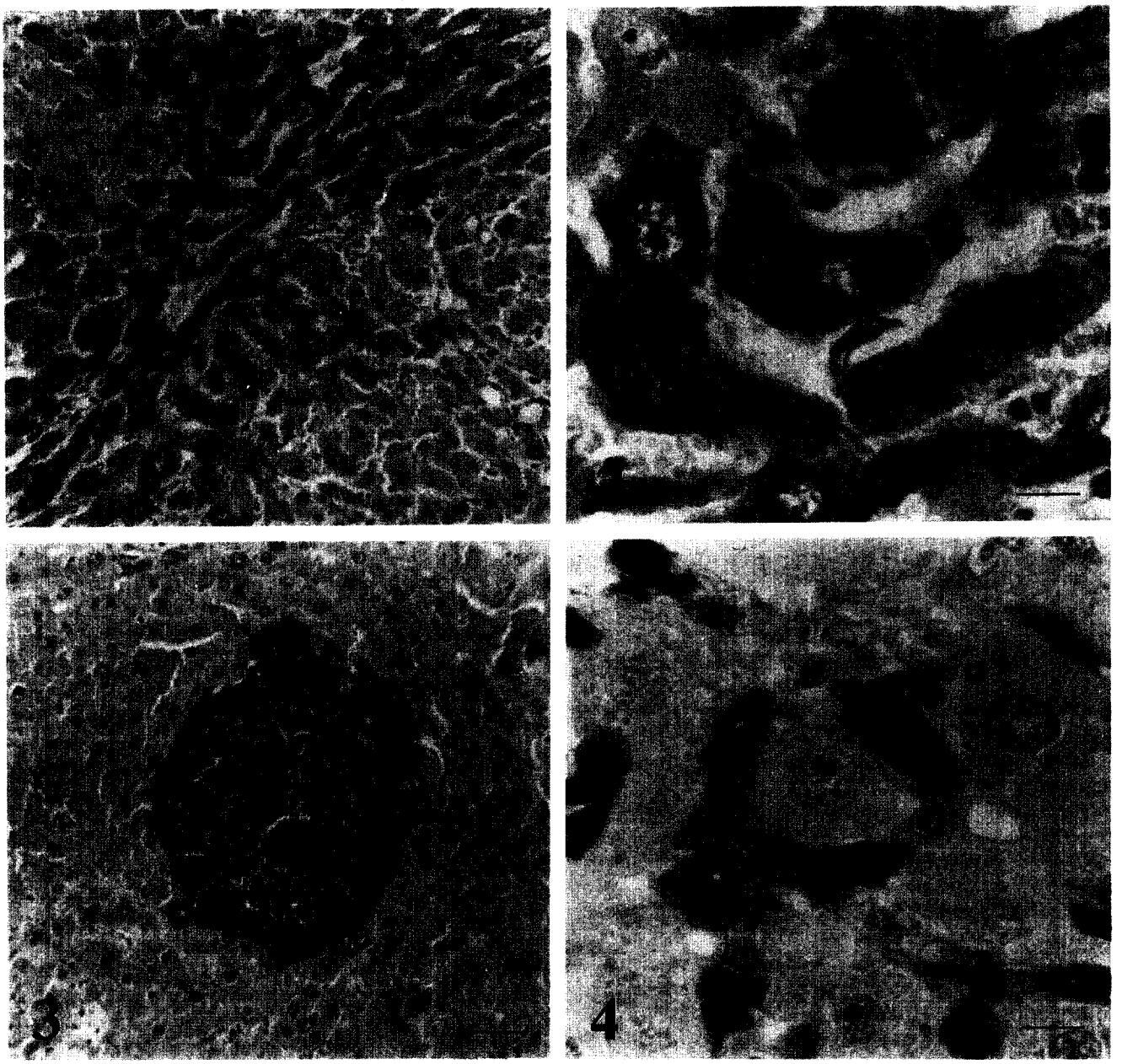


Fig.1. Liver; rat. Immunoreactivity for cytochrome P4502E1 was increased in degenerate hepatic cells in rat fed with juice and 4% pelleted diets containing *Petasites japonicus* for 12 weeks. Avidin-biotin-peroxidase method, Mayer's hematoxylin counterstain. Bar=16.6㎛.

Fig.2. Liver; rat. Higher magnification of fig. 1. Cytochrome P4502E1 highly expressed at intracytoplasm of hepatic cells. Avidin-biotin-peroxidase method, Mayer's hematoxylin counterstain. Bar=66.6㎛.

Fig.3. Liver; rat. GST-P positive preneoplastic foci demarcated with surrounding tissue were strongly stained in both nuclei and cytoplasm of rat fed with juice and 8% pelleted diets containing *Petasites japonicus* for 14 weeks. Avidin-biotin-peroxidase method, Mayer's hematoxylin counterstain. Bar=16.6㎛.

Fig.4. Liver; rat. Positive immunohistochemical staining of proliferative endothelial cells for GST-P in rat fed with juice of *Petasites japonicus* for 16 weeks. Avidin-biotin peroxidase method, Mayer's hematoxylin counterstain. Bar=66.6㎛.

Control: Three weeks old(n=10) and five weeks old(n=7) rats were fed a normal diet and water. Three weeks later, they were provided with water containing 0.05% PB.

Cytochrome P450 2E1 and GST-P immunohistochemistry

The interaction and enzyme induction were determined by immunohistochemical staining methods in rat liver using cytochrome P450s and GST-P.

After ether anesthesia, rats were sacrificed and their livers were rapidly removed. The tissues were fixed in 10% neutral formalin and Bouin reagent, dehydrated and paraffin-embedded. Endogenous peroxidase was blocked with 0.02% hydrogen peroxide/methanol and background staining decreased with 2.5% non-fat dry milk in phosphate buffered saline(PBS, pH7.4). Rabbit anti-GST-P (IgG/PBS/0.1% NaN_3) was supplied by Professor Kimihiko Satoh (Hirosaki University) and rabbit anti-cytochrome P4501A1/2 and 2E1 was supplied by professor James P. Hardwick (Northeastern University). Biotin-labelled goat anti-rabbit IgG and avidin-biotin-peroxidase complex were purchased (Sigma Chemical Company, St. Louis, MO, USA). The slides were incubated in a humidified chamber at room temperature followed by application of rabbit anti-cytochrome P4501A1/2 and 2E1 and rabbit anti-GST-P. Finally, the sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as peroxidase substrate and were counterstained with Mayer's hematoxylin.

Results

Immunohistochemistry for cytochrome P4501A1/2, 2E1, and GST-P was performed on the sections which were identified as degenerative hepatocytes and preneoplastic lesions histopathologically.

In the application of anti-cytochrome P450s to sections of liver, in all experimental groups CYP4502E1 immunoreactivity was increased in degenerative hepatocytes, approximately three to four times greater than normal hepatocytes, histopathologically (Fig. 1), but there was no expression of cytochrome P4501A1/2. Degenerative hepatocytes mixed with regenerative hepatocytes were stained with homogenously throughout the cytoplasm(Fig. 2).

After 12 weeks in all experimental groups, GST-P immunohistochemistry revealed strongly positive minifoci or even single cells in an early stage of hepatocarcinogenesis. GST-P positive preneoplastic foci demarcated with surrounding tissue, and were strongly stained in both the nuclei and cytoplasm. After 14 weeks, preneoplastic foci rats fed with juices and the 8% pelleted diet of *Petasites japonicus*(group 1) showed enhanced expression of GST-P(Fig. 3). After 16 weeks, GST-P positive preneoplastic foci were detected in rats fed with juices and 8% pelleted diet of *Petasites japonicus*(group 1) and in rats fed with juices(group 5), respectively. These progressed to large preneoplastic foci in a time-dependent manner.

In groups 1, 5 and 3(rats fed juices and 4% pelleted diet of *Petasites japonicus*), at 12 weeks GST-P immunoreactivity dominated in the hyperplastic endothelial cells which were immature cells form in the vascular space, often as small clefts, and in proliferative biliary epithelial cells after 12 weeks(Table 1)(Fig. 4)

. In all experimental groups which included group 4(rats fed 4% pelleted diet of *Petasites japonicus*), strong positive immunoreactivity of GST-P was detected in proliferative endothelial cells and biliary epithelial cells after 14 and 16 weeks and the incidence increased with time(Table 1).

Discussion

Hepatocarcinogenesis by genus *Senecio* has been studied in different species[5,10,14,40]. Cook et al.[10] have been reported liver tumors or nodules in rats fed an unspecified mixture of pyrrolizidine alkaloids from *Senecio jacobaea* for 8 months. It is thought that pyrrolizidine alkaloids, perhaps together with mycotoxins and viral agents such as hepatitis B, may contribute to liver cancer prevalent in certain areas[29]. Bull et al[5]. questioned whether pyrrolizidine alkaloids cause malignant tumors of hepatic parenchymal cells, since they interpreted the suspected hepatomas reported by Schoental et al.[40] as regenerating liver. It has been ambiguous to differentiate preneoplastic lesions from the foci of regenerating hepatocytes, histopathologically.

We performed a series of experiments on rats fed juices and pelleted diet with wild Korean native *Petasites japonicus* using the modified mid-term bioassay system. Altered hepatocytes have been visualized with GST-P[36,38,39] which apparently does not lead to positive staining of normal hepatocytes.

After GST-P positive diffuse single cells were observed at 12 weeks, further progressive preneoplastic foci were identified in liver after 14 weeks. After 12 weeks, proliferative endothelial cells and biliary epithelial cells stained positively. These data reinforce the experiment where rats fed diets containing the flower stalk of wild *Petasites japonicus* for 12~16 months developed hemangioendothelial carcinoma and hepatocellular carcinoma[16~18].

In this study, we observed preneoplastic foci in livers of rats fed juice and 8% pelleted diets of *Petasites japonicus* for 1 week and normal diet for 1 weeks alternately, and 8% pelleted diet. We did not detect preneoplastic foci in livers of rats fed juices and 4% pelleted diets, and 4% pelleted diets. But in all the experimental groups, GST-P positive minifoci or even single cells, and proliferative endothelial cells and billiary epithelial cells suggested an early stage of hepatocarcinogenesis. Therefore, we consider that further feeding may develop neoplasia as indicated by the response of GST-P positive minifoci, even single cells, proliferative endothelial cells, and billiary epithelial cells. These GST-P positive single cells, the number of which correlates with increasing dose of initiator in the mid-term bioassay system, using diethylnitrosamine(DEN)

as initiator are not induced by promoters of liver carcinogenesis(inducers of mixed- function oxidase) such as phenobarbital, methylcholanthrene, polychlorinated biphenyls and isosafrole. Therefore, these GST-P positive single cells should be considered as "initiated cells" with response to GST-P a very early marker.

Many chemical carcinogens undergo biotransformation to carcinogenically active metabolites via alteration by cytochrome P450 isoenzymes[1]. Cytochrome P4502E1 is a microsomal P450 enzyme normally expressed in liver and some extrahepatic tissues[42]. Among the various isozymes, cytochrome P4502E1 has been examined intensively with more than 80 compounds because of its capacity to metabolize drugs, solvents and environmental procarcinogens to cytotoxic compounds, and carcinogenic metabolites[24].

Chemical carcinogens evoke an altered expression of cytochrome P450 in the induced status[46]. Cytochrome P4501A1/2-associated mono-oxygenase activity has been identified in several cancer models[25,27]. Cytochrome P4501A1/2 is highly expressed in the preneoplastic lesions which were converted into phenotypically altered foci, by treatment with chemical carcinogens, DEN and 2-acetylaminofluorene[11]. These phenotypic alterations in cytochrome P450 and GST-P in tumors result in decreased ability to activate xenobiotics and increased capacity for xenobiotic detoxification by conjugation reactions[12]. *Petasites japonicus*, following partial hepatectomy, gave rise to increased immunoreactivity of cytochrome P4502E1. These results suggested that the increased level of cytochrome P4502E1 in affected hepatocytes was a direct consequence of *Petasites japonicus* toxicity. Futher, immunoreactivity to anti-GST-P in hepatocytes, endothelial cells and biliary epithelial cells suggested the carcinogenicity of Petasites japonicus in the Sprague-Dawley rat.

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