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Rat liver nodules induced by 2-acetylaminofluorene lose an ability to take up indocyanine green in the process of hepatocarcinogenesis.

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Abstract

Indocyanine green (ICG) was injected into rat liver nodules induced by 2-acetylaminofluorene (2-AAF) via portal vein. The relationship between ICG staining and cell atypism of liver nodules was examined by means of histology and DNA flow cytometry. After 2-AAF administration, many small nodules appeared on the liver surface. All hyperplastic nodules were ICG stained until 10 weeks after the administration, but some nodules were not stained after 14 weeks. ICG-stained nodules histologically consisted of benign tissues and borderline lesions, and many of them showed "diploidy" in DNA cytometry. ICG-unstained nodules consisted of hepatocellular carcinoma (HCCs) and borderline lesions, and many of them showed "aneuploidy". In this way, it has been suggested that HCC could derive from hyperplastic nodules and that they might lose an ability to take up ICG in the process of hepatocarcinogenesis. Immunohistochemical staining for glutathione-S-transferase alpha (GST-alpha), a carrier protein of ICG in hepatocytes, was well correlated with ICG staining in the nodules, suggesting that the loss of ICG uptake in HCC was partly due to the decrease of GST-alpha. Moreover, the appearance of ICG unstained and aneuploid nodules was significantly inhibited in rats which were fed on diet containing Syosaiko-to after the administration of 2-AAF. Chemopreventive effect of Syo-saiko-to on hepatocarcinogenesis was identified.

KEYWORDS: hepatocellular carcinoma, idocyanine green, carcinogenesis, DNA flow cytometry, Syo-saiko-to, glutathione-S-transferase

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Indocyanine green (ICG) was injected into rat liver nodules induced by 2-acetylaminofluorene (2-AAF) via portal vein. The relationship between ICG staining and cell atypism of liver nodules was examined by means of histology and DNA flow cytometry. After 2-AAF administration, many small nodules appeared on the liver surface. All hyperplastic nodules were ICG stained until 10 weeks after the administration, but some nodules were not stained after 14 weeks. ICG-stained nodules histologically consisted of benign tissues and borderline lesions, and many of them showed "diploidy" in DNA cytometry. ICG-unstained nodules consisted of hepatocellular carcinoma (HCCs) and borderline lesions, and many of them showed "aneuploidy". In this way, it has been suggested that HCC could derive from hyperplastic nodules and that they might lose an ability to take up ICG in the process of hepatocarcinogenesis. Immunohistochemical staining for glutathione-S-transferase alpha (GST-alpha), a carrier protein of ICG in hepatocytes, was well correlated with ICG staining in the nodules, suggesting that the loss of ICG uptake in HCC was partly due to the decrease of GST-alpha. Moreover, the appearance of ICG unstained and aneuploid nodules was significantly inhibited in rats which were fed on diet containing Syo-saiko-to after the adminstration of 2-AAF. Chemopreventive effect of Syo-saiko-to on hepatocarcinogenesis was identified.

Key words : **hepatocellular carcinoma, indocyanine green, carcinogenesis, DNA flow cytometry, Syo-saiko-to, glutathione-S-transferase**

It is sometimes difficult to diagnose small hepatocellular carcinomas (HCCs) only by routine histopathological findings since they are usually well differentiated. Therefore, new diagnostic devices would be useful. It has been reported that peritoneoscopic examination after the injection of a large dose of indocyanine green (ICG) is useful to detect HCCs on the liver surface (1, 2). We applied this method to ultrasound-guided liver biopsy (USGB) and reported that ICG staining of biopsy specimens was available as quick and functional diagnostic criteria of small and well differentiated HCCs (3, 4). Namely, ICG unstained tissues proved to be malignant or premalignant lesions, consisting of a majority of HCCs, metastatic liver tumors or adenomatous hyperplasia as a preneoplastic state although only a few exceptional HCCs were recognized in ICG stained tissues. It is still unknown why normal liver cells take up ICG organ-specifically and why

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malignant cells do not, although various studies have been done on the mechanism of ICG uptake into hepatocytes (5–15). ICG uptake by liver nodules induced by experimental carcinogenesis has been examined, and it was reported that HCCs were ICG unstained, while some hyperplastic nodules were weakly stained (16).

In this investigation, we injected ICG into rat liver nodules induced by 2-acetylaminofluorene (2-AAF) via portal vein and studied the relationship between ICG staining and cell atypism of liver nodules by means of histology and DNA flow cytometry. This procedure has recently received attention as a new diagnostic method for malignant tumors (17,18). Immunohistochemical staining for glutathione-S-transferase alpha (GST-alpha), reported to be a carrier protein of ICG in hepatocyte cytoplasm (19), was also performed in order to know the mechanism of losing ICG uptake in HCCs. Syo-saiko-to, a biological response modifier, has been used for prevention of HCC development (20, 21). Therefore we fed rats on diet containing Syo-saiko-to and examined its preventive effects on malignant transformation of nodules with respect to ICG staining in the process of hepatocarcinogenesis.

Materials and Methods

Administration of 2-AAF and Syo-saiko-to. According to the report by Epstein *et al*, (22); 36 Wister-strain male rats (8-week-old, 170–180g) were fed on a basal diet containing 0.05 % 2-AAF for the period of 0–3, 4–6, and 8–13 weeks. The basal diet without 2-AAF was fed intermittently for the period of 3–4 and 6–8 weeks. After the administration of 2-AAF, survivors were weighed and divided into two groups in order of body weight to avoid the influence of weight difference on the number of nodules produced. Each group was subsequently fed on a basal diet containing 1.22 % Syo-saiko-to (Group A) or basal diet alone (Group B) for the period of 13–26 weeks (Table 1).

ICG injection and histological diagnosis. Rats were sacrificed in order of weight at the time shown in Table 2. ICG was injected as follows: the rats were anesthetized with ether and the portal vein was canulated with a 24 gauge thin needle. A small dose of ICG (2.5 mg/ml) was injected slowly without giving pressure until the liver surface was generally stained green. Five minutes later, the inferior vena cava was incised at juncture with the hepatic vein, and the liver was perfused slowly with 20 ml of saline. Then, we examined the ICG staining of nodules visible from the surface of the liver, and the largest ICG stained or unstained nodules were regarded as objects in this investigation. Samples were cut into small pieces with a razor, fixed in 10 % neutral buffered formalin and embedded in paraffin. Every specimen was stained with hematoxylin-eosin. Azan-Mallory and periodic-acid-Schiff staining were also performed.

DNA flow cytometry. Small pieces of unfixed fresh frozen specimens were provided for DNA flow cytometry, and the nuclear DNA content was examined as follows: about 200 mg of tissues were minced finely with scissors and 5 ml of 0.1 % RNase, 0.1 % Triton X -100, 0.1 M phosphate buffered saline, pH 7.4 was added. After vortex mixing, the solution was filtrated with a 40 μm nylon mesh. Thereafter, the same volume of 100 μg/ml propidium iodide solution was added, and mixing and filtration were repeated. The final solution was examined with a FACScan analyzer (Becton-Dickinson Co. Ltd). The results were demonstrated as DNA histograms. Normal human peripheral blood mononuclear cells were used as controls. DNA index (DI) was calculated as follows: DI = G_0G_1 (1st) peak channels of sample/G_0G_1 (1st) peak channels of control. "Diploidy" and "aneuploidy"were defined as DNA histograms satisfying the following instances: 0.95 < 1st peak DI < 1.05 and 1st peak DI $\leqq$ 0.95 or $\geqq$ 1.05, respectively. However, if DNA histograms showed any other G_0G_1 peaks, they were judged as aneuploidy except the following instance: 1.95 < 2nd peak DI < 2.05, because

Table 1 Dietary regimens for administration of 2-AAF and Syo-saiko-to

Time (weeks)	Diets	
0 — 3	0.05 % 2-AAF	
3 — 4	Basal diet	
4 — 6	0.05 % 2-AAF	
6 — 8	Basal diet	
8 — 13	0.05 % 2-AAF	
	Group A	Group B
13 — 26	1.22 % Syo-saiko-to	Basal diet

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such 2nd peak might show proliferating G_2M peaks (so called "tetraploidy"). Moreover, if coefficient of variation (CV) value was more than 10% in each peak, or total cell count was less than 10,000, DNA histogram was not evaluated.

GST-alpha staining of liver nodules. Anti GST-alpha polyclonal antibody was raised in a rabbit. Formalin-fixed and paraffin-embedded sections of rat liver nodules were used. After dewaxing and dehydrating, immunohistochemical staining for GST-alpha in the nodules was performed with Histofine ABC kits (Nichrei Co. Ltd).

Statistical analysis. Differences in variable parameters between the two groups were evaluated using χ^2-test, and the mean values were compared with Student's t-test.

Results

Macroscopic findings. During the 7 weeks after the start of 2-AAF administration, 9 rats died of hunger (mortality rate = 9/36 = 25%). The smallest rat in 27 survivors was sacrificed at 7 weeks. Many small nodules of about 2mm in diameter appeared on the liver surface. Thereafter, we sacrificed the animals in order of body weight as shown in Table 2. Numerous nodules appeared on and in all 27 rat livers. We had an impression that ICG unstained nodules were more whitish and hard than ICG stained ones before ICG injection. However, it was not certain to decide which nodules were ICG stained or unstained before ICG injection. ICG stained nodules were clearly distinguishable from unstained ones after ICG injection as shown in Fig. 1. All nodules induced in 3 rat livers harvested from 7 to 10 weeks were stained with ICG. An ICG unstained nodule initally appeared at 12 weeks, and thereafter both ICG stained and unstained nodules developed. Histological diag-

Table 2 Time table for sacrifice of rats

Time (weeks)	Numbers of sacrificed rats	
7	1	
9	1	
10	1	
12	2	
13	2	
	Group A	Group B
18	2	2
22	4	4
26	4	4

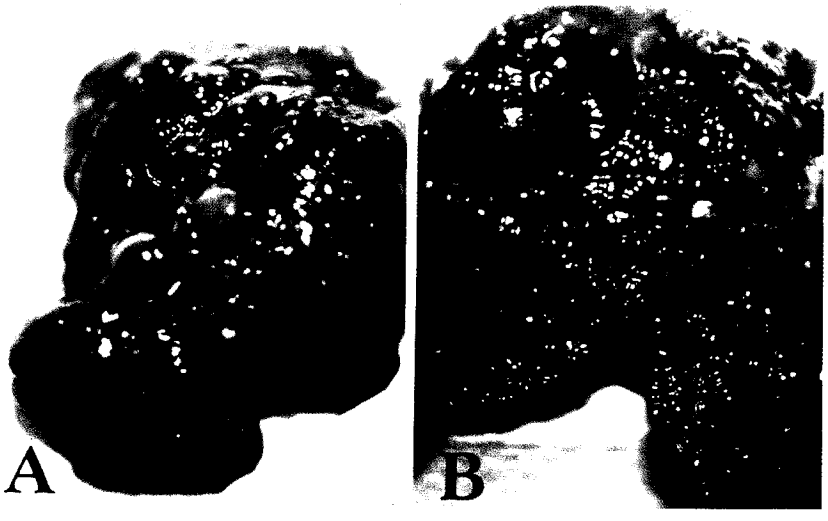

Fig. 1 Macroscopic findings of rat liver nodules after ICG injection.
A: ICG unstained nodule (arrow): The nodule was not stained with ICG although the surrounding tissue was stained. B: ICG stained nodule (arrow): The nodule was homogeneously stained with ICG.

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noses of all of the nodules examined were shown in Table 3.

Tumor sizes. Twenty-six ICG stained and 14 unstained nodules were obtained from 27 rats (Table 3). The largest nodule of each rat liver ranged from 2–20 (mm) in diameter in ICG stained nodules (mean = 9.0 ± 4.4) and 3–12 (mm) (mean = 7.9 ± 2.9) in unstained nodules respectively. These two groups did not differ significantly.

Histological findings. ICG stained nodules consisted of benign tissues (11/26, 42.3 %) and borderline lesions (15/26, 57.7 %) and were free from HCCs. In contrast, ICG unstained nodules consisted of HCCs (2/14, 14.3 %) and borderline lesions (12/14, 85.7 %) and were free from benign tissues. Cells in borderline nodules were swollen, clearized, and had large nucleoli. Some showed fatty deposits to various degrees and/or vacuolar changes of nuclei. In 2 HCCs, one was well differentiated with acinar arrangement, and the other was poorly differentiated with extreme

Table 3 Histological diagnoses of liver nodules

Hisology	Time of sacrifice (weeks)																					
	Group A							Group B														
	7	9	10	12	12	13	13	18	18	22	22	22	22	26	26	26	26	18	18	22	22	22	22	26	26	26	26
Benign	+	+	+		+	+		+		+	+			+		+		+									
Borderline				±	−	−	−	−	±			±	+		+		+		±	±	±	+	±	±	+	+	+
HCC[a]																		−									−

+: Indocyanine green (ICG) stained nodule, −: ICG unstained nodule, *a*: hepatocellular carcinoma

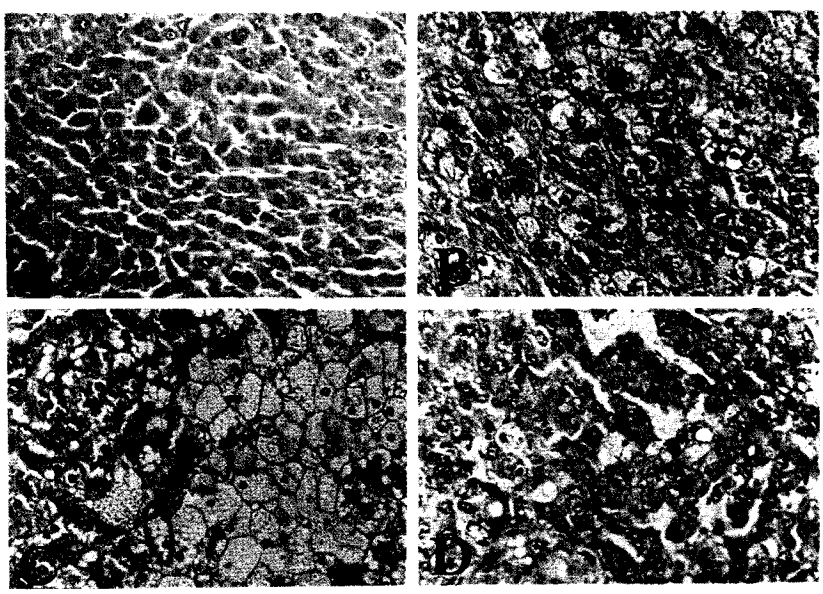


Fig. 2 Microscopic findings of rat liver nodules induced by 2-AAF.
A: ICG stained nodule (22 week): Benign tissue, resembling to normal rat liver. B: ICG unstained nodule (22 week): Borderline lesion. Clear cytoplasm and large nucleoli are remarkable, however, the nucleus/cytoplasm ratio is not so extreme. C: ICG unstained nodule (26 week): Borderline lesion. Clear cells and fatty changes are remarkable. D: ICG unstained nodule (26 week): Poorly differentiated HCC. Hypercellularity and large nucleus/cytoplasm ratio are recognized.

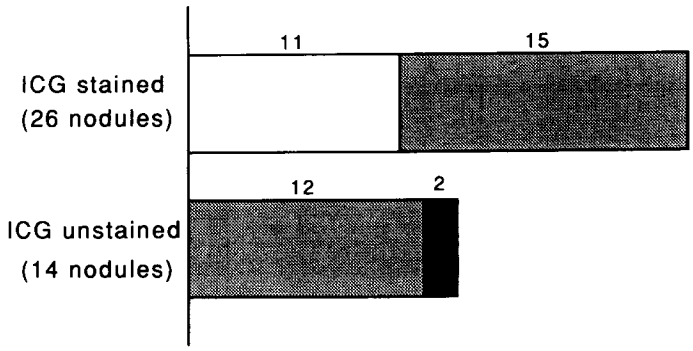


Fig. 3 Relationship between ICG staining and cell atypism of the liver nodules.
ICG stained nodules consist of benign tissues (□) (42.3 %) and borderline lesions (▒) (57.7 %), being free from HCCs (■), although ICG unstained nodules consist of borderline lesions (85.7 %) and HCCs (14.3 %), being free from benign tissues.

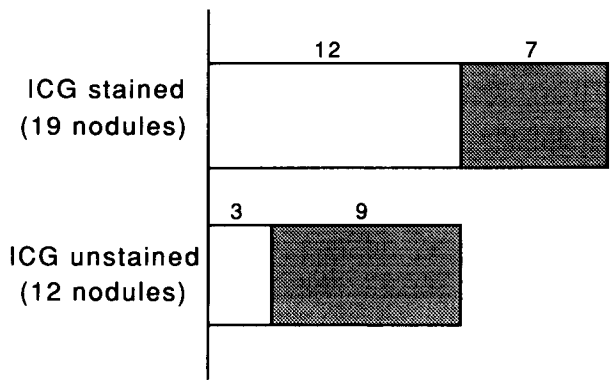


Fig. 4 Relationship between ICG staining and DNA ploidy pattern.
Diploidy (□) is dominant (63.2 %) in ICG stained nodules, while aneuploidy (▒) is dominant (75.0 %) in ICG unstained nodules ($p < 0.01$). ICG unstained aneuploid nodules contain two multiploid nodules.

hypercellularity and high nucleus/cytoplasm ratio. These microscopic findings were shown in Fig. 2. They resembled those reported by Farber *et al.* (23) or Teebor *et al.* (24). Both the borderline lesions and HCCs were significantly less in ICG stained than in unstained nodules ($p < 0.05$ and $p < 0.01$, respectively as shown in Fig. 3).

DNA flow cytometry. Nuclear DNA content analysis for all of the 40 nodules was performed by a FACScan analyzer. Nine nodules could not be evaluated because of cell destruction (8/40, 20.0 %) and large CV value (1/40, 2.5 %). The remaining 31 nodules represented a large S-phase rate compared with normal hepatocytes. As a result, 15 nodules (48.4 %) were judged as diploidy and 16 (51.6 %) as aneuploidy. In all aneuploid peaks, DI was distributed 0.94 − 2.82 (mean = 1.41). Diploidy was dominant in ICG stained (12/19, 63.2 %) compared with ICG unstained nodules (3/12, 25.0 %, $p < 0.01$). On the other hand, aneuploidy was dominant in ICG unstained nodules (9/12, 75.0 %) compared with stained nodules (7/19, 36.8 %, $p < 0.01$ as shown in Fig. 4). Characteristic DNA histo-

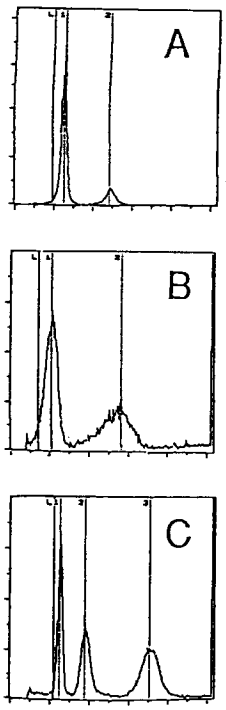
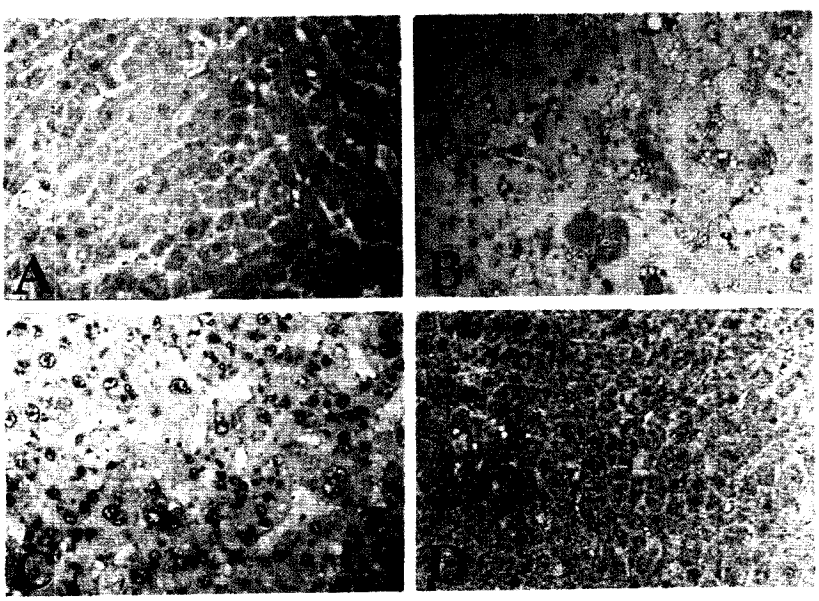


Fig. 5 (Left) DNA histograms of the liver nodules.
A: DNA histogram showing diploid pattern from an ICG stained nodule (22 week) which is shown in Fig. 2-A (1st peak DI = 1.00). B: Aneuploid pattern with a large S-phase rate observed in the ICG unstained nodule (22 week) which is shown in Fig. 2-B (2nd peak DI = 2.20). C: Multiploid pattern with triple G_0G_1 peaks observed in the ICG unstained nodule (26 week) which is shown in Fig. 2-D (2nd peak DI = 1.57, and 3rd peak DI = 2.82).

Fig. 6 (Bottom) GST-alpha staining of the nodules.
A: ICG stained benign tissue (22 week): GST-alpha is strongly positive in the cytoplasm of the nodule. B: ICG unstained borderline lesion (26 week); About half of the cells in the nodule are GST-alpha negative. C: ICG unstained HCC (26 week): GST-alpha is not stained in the nodule. D: ICG stained normal rat liver (7-week-old): GST-alpha is positive in the cytoplasm.



grams were shown in Fig. 5. Both well, and poorly differentiated HCCs prominently showed "multiploidy" which had triple G_0G_1 peaks.

GST-alpha staining of liver nodules. Early hyperplastic nodules were GST-alpha stained until 10 weeks after the start of 2-AAF

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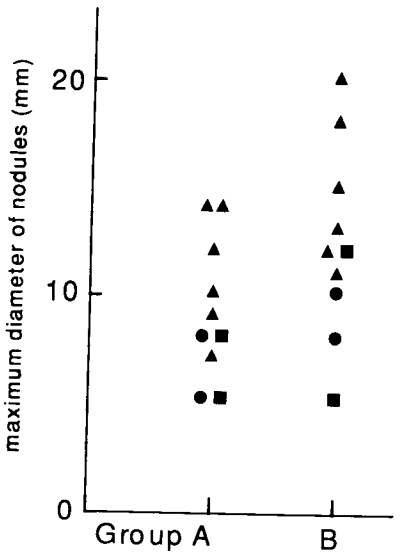

Fig. 7 Comparison of the maximum diameter of the nodules in Groups A and B.
Rats were sacrificed at 18 week (■), 22 week (●), and 26 week (▲). Group A is fed on basal diet containing 1.22% Syo-saiko-to and Group B is basal diet alone. Nodules in Group A are significantly smaller than in Group B ($p < 0.05$).

administration. However, some late hyperplastic nodules after 12 weeks were weakly stained or unstained for GST-alpha, and both of the 2 HCCs were negative for GST-alpha. ICG stained nodules were likely to be stained with GST-alpha (Fig. 6).

Effects of Syo-saiko-to. Each rat in the Groups A and B was sacrificed as shown in Table 2. Distribution of maximum diameter of the nodules was shown in Fig. 7. Nodules in Group A were significantly smaller than those in Group B ($p < 0.05$). ICG unstained nodules appeared in only 3 of 10 (30.0%) in Group A, compared with 7 of 10 (70.0%) in Group B ($p < 0.05$). Furthermore, 2 HCCs were induced only in Group B. As the result of DNA flow cytometry, diploidy was dominant in Group A (7/9, 77.8%)although aneuploidy was dominant in Group B (7/10, 70.0%). Aneuploidy was significantly more frequent in Group B than in Group A ($p < 0.05$).

Discussion

Biopsy specimens of small HCCs obtained from USGB are frequently well differentiated. Therefore, pathologists sometimes hesitate to conclude whether they are malignant or benign. We have developed a new diagnostic method called USGB-ICG: USGB after the injection of a large dose of ICG. It is a quick and functional method to diagnose small HCCs. Patients were injected intravenously with 2.0 mg/kg weight of ICG 10–20 min before USGB. Making use of an infrared camera, we could easily judge ICG staining of the specimens which we could not judge with the naked eye and reported that ICG unstained tissues might be functionally malignant (3, 4). In the current study, we injected a larger dose of ICG via portal vein and proved that ICG uptake of the nodules could be easily judged in the same way as peritoneoscopy. Therefore, this examination could be the experimental model of USGB-ICG.

Although all hyperplastic nodules until 10 weeks were ICG stained, some ICG unstained nodules appeared after 12 weeks by continuing 2-AAF administration. HCCs, borderline lesions and aneuploidy were significantly more frequent in ICG unstained than in stained nodules, suggesting that hyperplastic nodules might lose an ability to take up ICG in the process of hepatocarcinogenesis. Borderline lesions which were histologically diagnosed consisted of both ICG stained and unstained, or both diploidy and aneuploidy. These respective nodules were supposed to represent the different stages of carcinogenesis. In our study, only 2 HCCs appeared. It may be due to the shortening of 2-AAF administration (for 10 weeks), because 2-AAF administration for 3 months rarely produced HCCs in contrast with much higher incidence in rats administrated for 4 months (24).

Initially, we were concerned that ICG staining was affected by the disorder of blood flow into liver nodules according to their growth. The results did not show a significant relationship

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between ICG staining and the sizes of nodules in this experiment. Therefore, ICG staining might really depend on liver cell functions instead of the effect of blood flow or injection pressure. If all of HCCs derive from ICG stained hyperplastic nodules in rats, ICG unstained nodules should be larger than ICG stained ones according to their growth. However, this experiment showed that this was not the case, suggesting HCCs were not only generated from hyperplastic nodules, but also *de novo*.

Hepatocellular uptake of organic anions has been well investigated, and the receptor on the liver plasma membrane (5–7) or active transport mechanism (8–10) are supposed to regulate the transport of sulfobromophthalein (BSP), bilirubin and ICG into hepatocytes. Wolkoff *et al.* (25) have reported that BSP receptor is a single protein of 55 kd. On the contrary, BSP and bilirubin are supposed to be taken up into hepatocyte through the albumin–albumin receptor complex of the liver plasma membrane (11–13). However, the mechanism of ICG entry into hepatocytes has not be identified yet, and it remains unknown why uptake of ICG is not observed in HCCs. Malignant cells might lose ICG binding protein on the liver plasma membrane in the process of carcinogenesis, as the asialoglycoprotein receptor (26).

On the other hand, GST-alpha, which is a carrier protein of ICG in hepatocyte cytoplasm, might play an important role in ICG staining (14, 15, 16, 27, 28). It has been reported that GST-alpha functions as "storage" for organic anions taken up into hepatocytes (14) or that 2-AAF is detoxicated by combining with GST-alpha and excreted into bile (29). In practical studies, it was supposed that GST-alpha in human hepatocytes acted as "ligandin" of ICG since ICG staining pattern on peritoneoscopy was identified with the immunohistochemical distribution of GST-alpha in advanced liver injuries (27). The decrease of GST-alpha in HCCs was also indicated by Sherman *et al.* (30). With respect to our experiment, GST-alpha was well stained in ICG positive nodules, and ICG unstained HCCs were GST-alpha negative, suggesting that the loss of ICG uptake in HCCs is partly due to the decrease of GST-alpha.

Recently, nuclear DNA content for solid tumors has been commonly analyzed with flow cytometry. However, there are only a few reports for HCC and no consent to the practical significance of its ploidy patterns (31–35). It has been reported that vascular invasion, intrahepatic metastasis and subsequent poor prognosis are more frequent in aneuploid HCCs than in diploid HCCs (31, 32). However, another report contends that ploidy patterns are not directly related to the prognosis (33). In our examination, both ICG stained and unstained nodules represented large S-phase rate, diploidy being dominant in ICG stained and aneuploidy being dominant in ICG unstained nodules. This indicated that ICG stained nodules contain the regenerating or proliferating cells and that cells in the ICG unstained have already become neoplastic, although they are equally diagnosed as "borderline" by histological findings. Aneuploidy probably represents malignant cell proliferation in experimental hepatocarcinogenesis. Curiously 2 clearly diagnosed HCCs showed multiploidy, which had triple G_0G_1 peaks, suggesting the presence of at least three clones with different DNA contents in HCCs induced by 2-AAF.

One of the purposes of this study is to evaluate the effects of Syo-saiko-to on the liver nodules. Glycyrrhizin, one of its constituents, inhibited the promotion of mouse skin cancer induced by teleocidin (36), and a chemopreventive effect of Syo-saiko-to in 2-AAF induced HCC was reported by Okita *et al.* (20). In our study, only 2 HCCs (20.0 %) were induced in Group B. This incidence was low as compared to Okita's report (55.6 %). It is difficult to conclude hastily that Syo-saiko-to is effective in prevention of HCC development because of the low incidence. However, ICG unstained and aneuploid nodules were significantly more frequent in Group B. With respect to these findings,

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it is possible to say that Syo-saiko-to might prevent hepatocarcinogenesis and would therefore be useful as a biological response modifier (37). Further basic and clinical studies into the individual principles (*e.g.*, glycyrrhizin or saiko-saponin) can be expected to be requested in the future.

In conclusion, USGB-ICG may be well available as a quick and functional diagnostic method complementary to histological diagnosis of small HCCs. This method should be used for the tissues on which histological diagnosis seems to be difficult. However, it is still unknown why HCCs do not take up ICG, as the theoretical ground of USGB-ICG. Moreover, a few HCCs proved to be ICG stained on the clinical study. Further study on the distribution of organic anion binding proteins and GST-alpha in reference to the cell atypism is now in progress in our laboratory.

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