

Effects of Iodinated Fatty Acid Ester on Human Hepatocellular Carcinoma Cells

Hoguen Kim¹, Chanil Park², Yoo Bock Lee²,
Hyung Sik Yoo³, Jeon Han Park⁴ and Won-Young Lee⁴

The interaction between Lipiodol and cells was studied by treating Lipiodol in a human hepatocellular carcinoma cell line(Hep) and mouse fibroblast cell line(L929). Irregular, sustained radioactivity was released from both cell lines shortly after incubation in the radioiodinated Lipiodol mixed media. Lipiodol droplets were found to be firmly attached to the cells following the incubation and these cells were strongly positive for fat stains. The radioiodinated Lipiodol demonstrated the same behavior of accumulation within the cell and on the cell membrane. Although the amount of Lipiodol attached was almost equal in both of the cell lines, the final amount accumulated in the cells was larger in the Hep cells. The accumulation of Lipiodol within the cell and on the cell membrane may play a significant role for its selective targeting and its prolonged retention in the solid tumor.

Key Words: Lipiodol, I-131-Lipiodol, hepatocellular carcinoma

Iodinated oil, such as Lipiodol, has been known to be selectively retained in hepatocellular carcinoma (HCC) for a long time following intraarterial hepatic injection (Bookstein 1985; Yumoto *et al.* 1985). Based on this finding, Lipiodol is now successfully used for the detection of small sized HCC and daughter nodules as well as the differentiation from other benign neoplasms (Nakakuma *et al.* 1985; Ohnishi *et al.* 1985). In addition, Lipiodol is used as a carrier of lipophilic anticancer drugs for selective drug targeting into the tumor tissue and this method has proved to be of therapeutic value (Kono *et al.* 1983 & 1984; Ohnishi *et al.* 1984; Okayasu *et al.* 1988). Recently, a new approach using a radioiodinated Lipiodol (I-131-Lipiodol) in

the treatment of HCC was reported (Yoo *et al.* 1986) and this method also proved to be of good therapeutic and diagnostic value in the treatment of patients with HCC, especially the expanding type of HCC sized less than 8cm (Yoo *et al.* 1988; Park *et al.* 1990).

The mechanism of selective retention of this contrast material in the tumor cells is not clearly proved, although there are several proposed mechanisms (Iwai *et al.* 1984; Kono *et al.* 1984; Park *et al.* 1990). In addition, the exact histologic sites and the essential events of this long retention period in the tumor area are still obscure. Moreover, the intracellular accumulation of Lipiodol and the amount of Lipiodol essentially required for the selective drug delivery are not clearly elucidated.

In this study, we intended to clarify the interaction of Lipiodol with cultured benign and malignant cells, which might explain the mechanism of selective and long-term retention of Lipiodol in HCC.

MATERIALS AND METHODS

Cell lines

An established human hepatocellular carcinoma

Received June 15, 1990

Accepted September 10, 1990

Department of Pathology¹, Wonju Medical College Yonsei University, Wonju, Korea

Departments of Pathology², Radiology³, and Microbiology⁴, Yonsei University College of Medicine, Seoul, Korea

This study was supported by a Yonsei University Research Grant.

Address reprint requests to Dr. H Kim, Department of Pathology, Wonju Medical College Yonsei University, 162 Ilsan-Dong, Wonju, Kangwon-Do, Korea, 220-701

cell line, Hep, and a continuous cell line of mouse fibroblasts, L929, maintained in our Institute laboratory at Yonsei University were used. Both cell lines were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum in a 5% CO₂ atmosphere.

Preparation of lipiodol-containing culture media

The Lipiodol (Andrea-Gelbe Laboratories, France) was mixed with MEM at a concentration of 1:30. The mixture was thoroughly mixed by use of double syringes. The final product was a transparent, oily, homogeneous, emulsified liquid (Fig. 3). The radioiodinated Lipiodol (I-131-Lipiodol) was mixed by the same method. In this experiment, 1ml of I-131-Lipiodol with 4mCi was used.

Radioiodination of lipiodol

Radioiodination of Lipiodol was achieved in the Isotope Division, Korea Advanced Energy Research Institute by a simple exchange method (Yoo et al. 1986 & 1988). Lipiodol has 30% iodine (I-127) by weight. High specific activity I-131 was boiled dry in 0.1M NaOH to dry in the presence of 0.5mg of potassium iodide. The residue was refluxed in 25ml of acetone for 20 minutes, and then 1-2ml of Lipiodol was added. The solution was again refluxed for another 30 minutes. The acetone was removed using a rotating evaporator in a 70 °C water bath. The cooled residue was sterilized by boiling in butanol. The labeling efficiency was greater than 99% which was confirmed by paper chromatography, and the agent was found to be stable in vivo.

Lipiodol uptake assay

Radioactivity Assay for I-131-Lipiodol Laden Cells: Following the incubation of 5×10^3 from both cell lines were cultured in the chamber slides (Lab Tec, Illinois) filled with the mixture of MEM and Lipiodol. After the various incubation times mentioned above, the cells were washed thoroughly 3 times with the fresh MEM. Then the cells were fixed with 95% alcohol for 20 minutes. After fixation, routine hematoxylin-eosin staining was performed. The total number of cells and the number of cells with Lipiodol accumulation were calculated under the light microscope. Three replicated chambers were made for each assay. One of the slides was stained with Sudan black B or Oil red O without fixing for the direct demonstration of Lipiodol.

RESULTS

Morphological changes of the cultured cells

The mixture of MEM and Lipiodol was well emul-

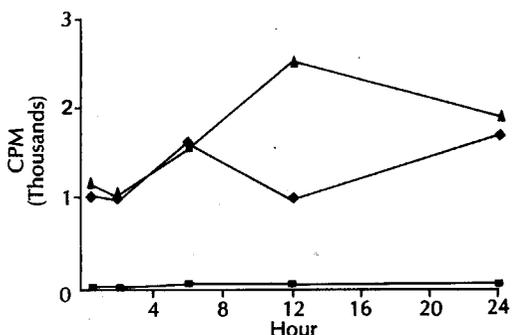


Fig. 1. Radioactivity released by Hep cells(▲) and L929 cells(◆) incubated in I-131-Lipiodol-containing medium. Both cell lines showed irregularly increased radioactivities shortly after the incubation with I-131-Lipiodol-containing medium. A small amount of spontaneous radioactivity was noticed in the Hep cells incubated with I-127-Lipiodol-containing medium(■).

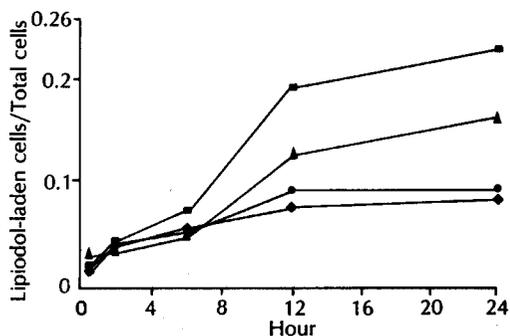


Fig 2. The proportion of Lipiodol-laden Hep cells and L929 cells incubated in Lipiodol or I-131-Lipiodol-containing medium.

- ▲: Hep cells incubated in I-131-Lipiodol-containing medium.
- : Hep cells incubated in Lipiodol-containing medium
- ◆: L929 cells incubated in I-131-Lipiodol-containing medium
- : L929 cells incubated in Lipiodol-containing medium

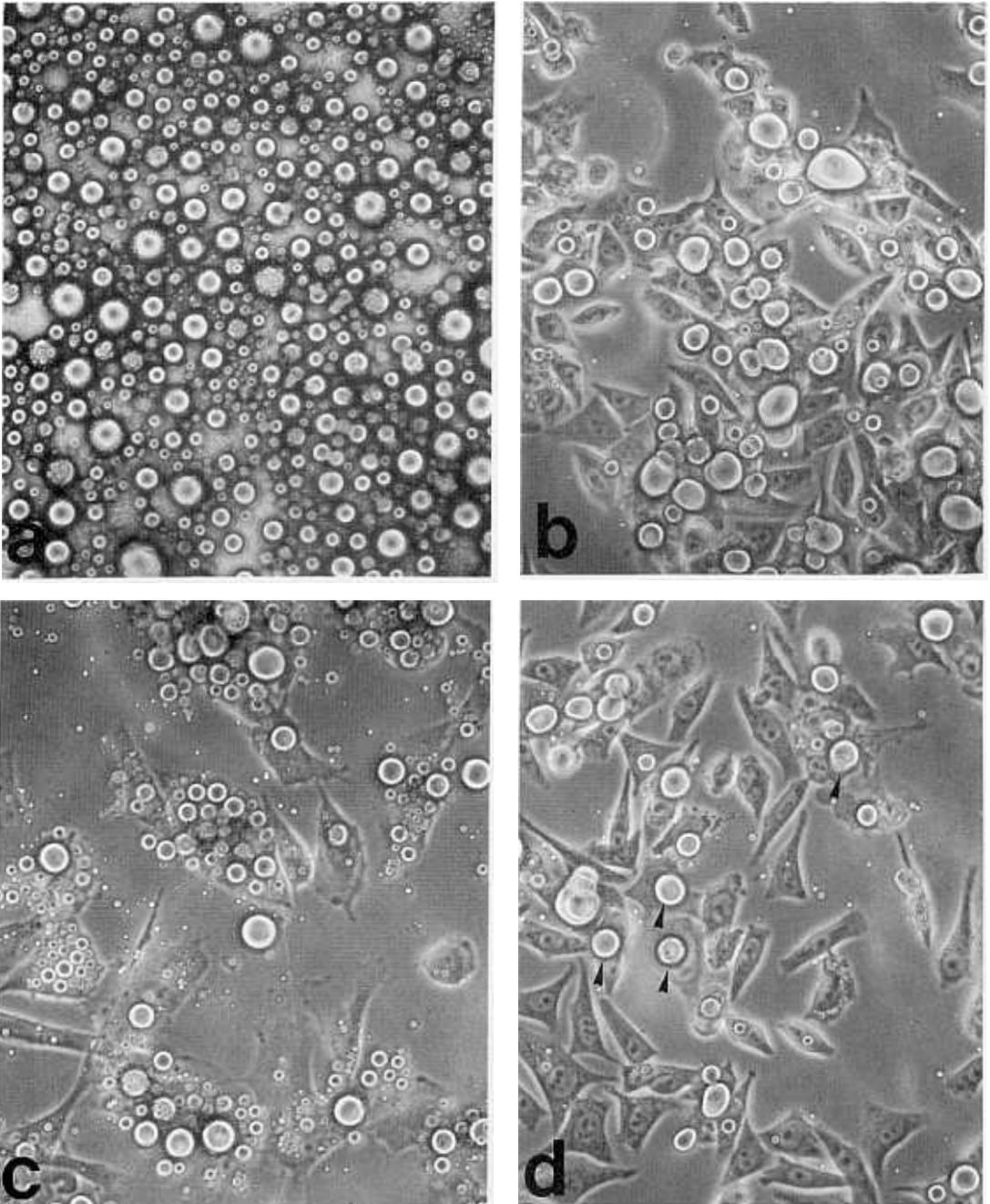


Fig. 3. Photomicrograph of cultured cells. a) Lipiodol-containing medium showing homogenously emulsified Lipiodol droplets over the growing cells ($\times 100$). b) Hep cells incubated in Lipiodol-containing medium showing a large amount of membrane attached Lipiodol droplets ($\times 100$). c) L929 cells incubated in Lipiodol-containing medium showing a large amount of membrane attached Lipiodol droplets ($\times 100$). d) Hep cells incubated in I-131-Lipiodol-containing medium showing intracellularly accumulated Lipiodol droplets. Most of the droplets are large-sized (arrowhead) and unilocular ($\times 100$).

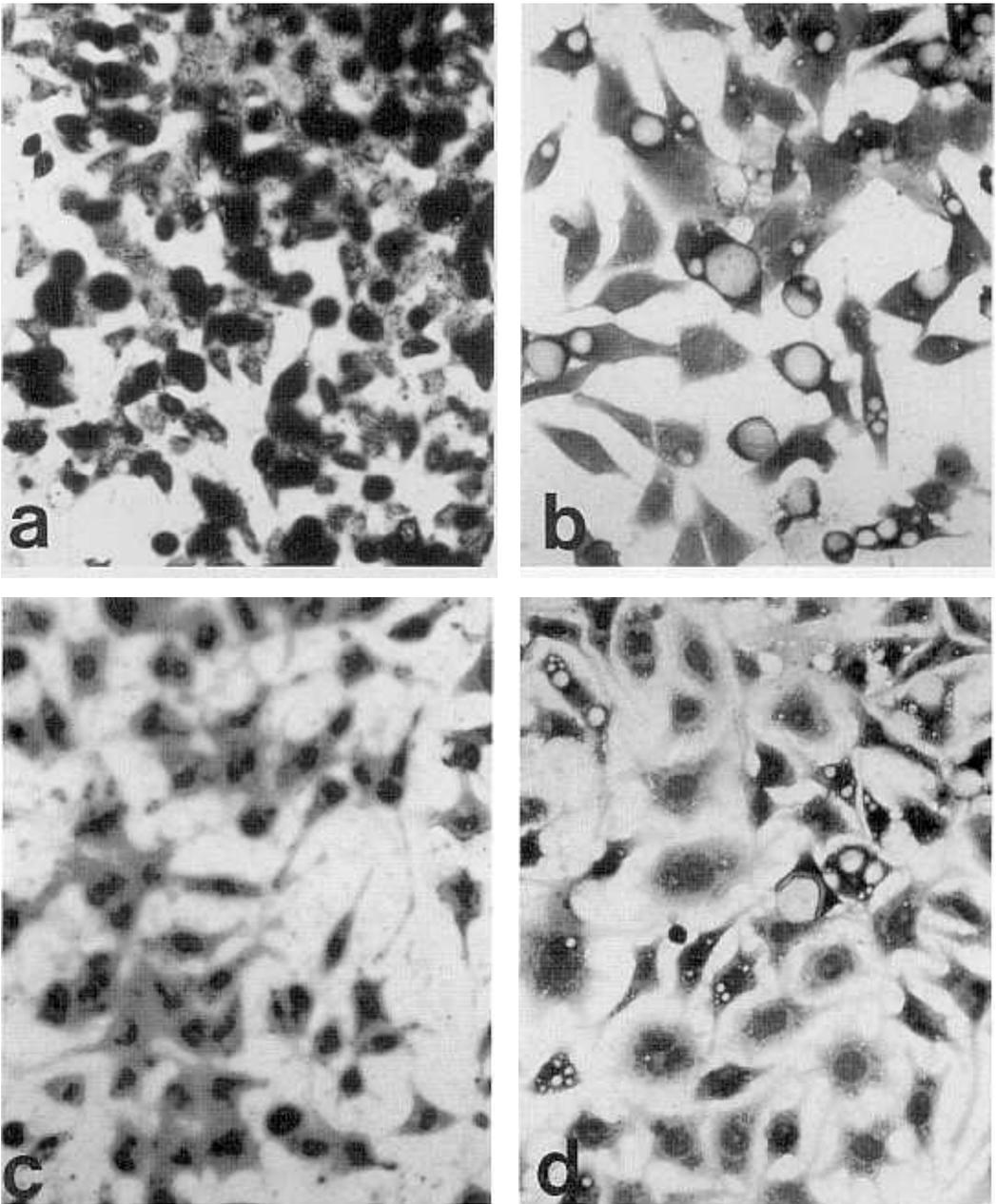


Fig. 4. Cytological and cytochemical features of the cultured cells. a) Sudan black B stain of the Hep cells incubated in Lipiodol containing medium showing a strong positive reaction to the membrane attached and intracytoplasmic Lipiodol droplets ($\times 100$). b) Hep cells incubated in Lipiodol-containing medium and fixed with 95% alcohol; most of the intracellularly located Lipiodol droplets appeared as large and unilocular empty vacuoles (H & E, $\times 200$). c) Oil red O stain of L929 cells incubated in Lipiodol-containing medium showing a strong positive reaction to the membrane attached and intracytoplasmic Lipiodol droplets ($\times 200$). d) L929 cells incubated in Lipiodol containing medium and fixed with 95% alcohol; most of the intracellularly located Lipiodol droplets appeared as multilocular vacuoles of various sizes (H & E, $\times 200$).

sified and evenly distributed in the culture slide chamber (Fig. 3a). Some of the Lipiodol droplets left firmly adhered to the growing cells after washing of the mixture medium by MEM (Fig. 3b & 3c). No remarkable morphologic differences between the Hep and L929 cells were observed according to the cell membrane were large enough and firm. However, a small portion of the cells were found to have intracellular droplets (Fig. 3d). The pattern and size of the intracellular Lipiodol droplets appeared to be somewhat different between the two cell lines (Fig. 4b & 4d). Most of the Lipiodol laden Hep cells were occupied with huge unilocular clear vesicles, whereas several intracellular clear vesicles of various size were noticed in the L929 cells. No intracellular vesicles were observed in both cell lines of the control group.

I-131-Lipiodol uptake by Hep and L929 cells

The mixture medium of MEM and I-131-Lipiodol

yielded constantly strong radioactivities throughout the experiment. The cells incubated with the medium showed only a small amount of spontaneously released radioactivity (Fig. 1).

From the cells incubated with MEM and I-131-Lipiodol, a strong radioactivity began to be detected shortly after the incubation. The difference in radioactivities between the two cell lines was not significant, even though the radioactivity seemed to be slightly higher in Hep cells than in L929 cells. Figure 1 shows a slight increase in radioactivity depending upon the incubation time regardless of the cell lines, but the pattern was very irregular.

Cytochemistry

In the cells incubated with the mixture of MEM and Lipiodol or I-131-Lipiodol, the intracytoplasmic or membrane-attached vesicles revealed a strong positive reaction to Sudan black B and Oil red O stains (Fig. 4a & 4c). Lipid droplets were not found

Table 1. Effect of Lipiodol and radioiodinated Lipiodol mixed medium on the Hep cells

Group	Control Medium		I - 127-Lipiodol mixed Medium		I - 131-Lipiodol mixed Medium	
	No. of total cells ($\times 10^{-2}$)	No. of lipid laden cells ($\times 10^{-2}$)	No. of total cells ($\times 10^{-2}$)	No. of lipid laden cells ($\times 10^{-2}$)	No. of total cells ($\times 10^{-2}$)	No. of lipid laden cells ($\times 10^{-2}$)
0.5	35.0 \pm 6.2	-	33.1 \pm 2.8	0.7 \pm 0.4	30.7 \pm 0.8	0.9 \pm 0.5
2	34.6 \pm 1.8	-	32.9 \pm 1.9	1.5 \pm 0.5	30.7 \pm 1.5	1.1 \pm 0.4
6	33.3 \pm 6.5	-	35.5 \pm 5.1	2.6 \pm 0.5	28.9 \pm 5.6	1.5 \pm 0.2
12	39.2 \pm 5.8	-	37.9 \pm 1.7	7.3 \pm 0.8	31.2 \pm 0.5	3.9 \pm 0.6
24	42.2 \pm 6.1	-	37.2 \pm 4.5	8.7 \pm 3.3	30.8 \pm 2.8	5.0 \pm 1.3

Values are Mean \pm S.D., (n=3)

Table 2. Effect of Lipiodol and radioiodinated Lipiodol mixed medium on the L929 cells

Group	Control Medium		I - 127-Lipiodol mixed Medium		I - 131-Lipiodol mixed Medium	
	No. of total cells ($\times 10^{-2}$)	No. of lipid laden cells ($\times 10^{-2}$)	No. of total cells ($\times 10^{-2}$)	No. of lipid laden cells ($\times 10^{-2}$)	No. of total cells ($\times 10^{-2}$)	No. of lipid laden cells ($\times 10^{-2}$)
0.5	36.8 \pm 6.1	-	36.6 \pm 4.1	0.7 \pm 0.2	35.2 \pm 3.8	0.7 \pm 0.4
2	39.2 \pm 1.1	-	38.8 \pm 3.3	1.5 \pm 0.6	33.5 \pm 2.9	1.4 \pm 0.8
6	37.0 \pm 1.2	-	35.1 \pm 4.3	1.9 \pm 0.9	30.2 \pm 5.6	1.6 \pm 0.5
12	38.9 \pm 3.3	-	36.9 \pm 2.4	2.9 \pm 0.3	32.5 \pm 2.8	3.1 \pm 0.4
24	43.3 \pm 4.6	-	44.8 \pm 3.8	3.8 \pm 0.4	39.2 \pm 4.5	3.6 \pm 0.5

Values are Mean \pm S.D., (n=3)

regardless of the incubation time in cells of the control group. No difference in the stain pattern between Lipiodol and I-131-Lipiodol was noticed.

Proportion of lipiodol harboring Hep and L929 cells

When the cells were fixed with 95% alcohol and stained with hematoxylin and eosin, all lipid droplets were dissolved and only the intracytoplasmic lipid droplets were left as clear empty vacuoles in the cytoplasm. The number of total cells and Lipiodol-containing cells were counted (Table 1 & 2). When incubated in the mixture of MEM-containing Lipiodol or I-131-Lipiodol, the total number of cells was slightly decreased in comparison to the control group, and it was especially obvious when the cells were treated with I-131-Lipiodol. However, morphological evidence of cytotoxicity was not apparent. When incubated in the mixture of MEM-containing Lipiodol and/or I-131-Lipiodol, the number of Lipiodol laden cells increased gradually according to the incubation time. And the proportion of Lipiodol laden Hep cells was greater than that of the L929 cells throughout the experiment and this was prominent after 12 hours of incubation (Table 1 & 2 and Fig. 2).

DISCUSSION

Reports indicating advantages of Lipiodol in both the diagnosis and the treatment of solid tumors are accumulating (Bookstein. 1985; Iwai *et al.* 1984; Kanematsu *et al.* 1984; Konno *et al.* 1984; Yoo *et al.* 1988). At present, Lipiodol is used for the treatment of HCC by delivering dissolved lipophilic anticancer drugs selectively to the target cells and by killing target cells with internal radiation by radioiodized Lipiodol (Fukushima *et al.* 1987; Konno *et al.* 1984; Yoo *et al.* 1986 & 1988).

The use of Lipiodol for selective drug delivery to tumors has been tried by Japanese scientists and success in experimental and clinical trials has been reported (Kono *et al.* 1983 & 1984; Ohnishi *et al.* 1984). The basic mechanism of the treatment is thought to be the combination of the long-lasting selective delivery of anticancer drugs and embolization by Lipiodol at the level of neo-vasculature in the tumor. In addition, many successful results of detection for small hepatoma and daughter nodules by Lipiodol injection in angiography have been reported (Bookstein. 1985; Iwai *et al.* 1984; Nakakuma *et al.* 1985; Ohnishi *et al.* 1984;

Yumoto *et al.* 1985).

One of the advanced therapeutic trials for HCC involving selective internal radiation therapy with radioiodinated Lipiodol via superselective catheterization of HCC feeding vessels has been clinically tried by one of our authors. The clinical results appear to be quite promising, especially in the expanding type HCC. In this type of HCC, more than 70% of the cases showed good clinical response. Even in the lowest responsive type of HCC, the infiltrative type, 10% of the cases showed some clinical response. Moreover, the accurate size determination and detection of daughter nodules were possible with the use of I-131-Lipiodol which persisted selectively in the HCC for a long period of time (Yoo *et al.* 1988). This result suggested that selective retention of the radioiodinated Lipiodol in the HCC, which was noticed by non-radioiodinated Lipiodol, was strong enough to produce effective internal irradiation in most of the HCC.

In spite of the numerous experimental and clinical evidence for the selective retention of Lipiodol in the HCC area, the mechanism and histological locations of this selective retention are not yet obvious. The mechanism of the selective effect on solid tumors is considered to be attributed to several factors. The highly leaky character of the tumor neovasculature, partly due to the hypervascular nature itself and partly due to the defective neural control of tumor vessels, is suggested to be responsible for this selective tumor retention. The lack of recovery of this macromolecular lymphographic agent due to the underdeveloped lymphatic system of the tumor may be another important factor (Iwai *et al.* 1984; Kono *et al.* 1984).

Although the architectural differences between the tumor and the normal tissue may play a major role in the prolonged retention of Lipiodol in the HCC, the possibilities and the roles of intracellular retention or cell membrane attachment should not be ignored. Iwai *et al.* (1984) found that Lipiodol, when injected into the normal liver of rabbits, was distributed in the entire arterial lumina, retained for about 24 hours, and gradually disappeared thereafter; but when injected into the liver with an implanted tumor, it was retained for a longer period of time. Moreover, Lipiodol was noticed in the tumor vessels as well as in the tumor cells by autoradiography. Recently we reported the distribution pattern of Lipiodol in the I-131-Lipiodol infused HCC. The Lipiodol was noticed in the cytoplasm as a globular lipid and in the cell surface as a nonglobular lipid (Park *et al.* 1990).

In this study we examined the amount of Lipiodol taken by the cultured HCC cells and fibroblasts. Lipiodol was found to have a high affinity for the cell membrane throughout the experiment. The attachment of Lipiodol to the cell membrane showed no specificity in the cultured HCC cells and fibroblasts. These findings may support the irregularly sustained high radioactivities of the cell fractions incubated with MEM-containing I-131-Lipiodol. Lipiodol may be attached to the cell membrane in vivo as well as in vitro, and the fact that Lipiodol was retained in the entire arterial lumina of the liver for about 24 hours (Iwai et al. 1984) may reflect that the attachment of Lipiodol to the endothelial cell membrane is transient. Lipiodol is a macromolecule, therefore, it is very unlikely that Lipiodol is evenly distributed in the tissue from the vessels. When Lipiodol leaks out of blood vessels in the tumor, retention of Lipiodol in tumor tissue for a long time can be expected because of the underdeveloped lymphatics of the tumor. The prolonged retention time may cause increased membrane attachment of Lipiodol, which offers advantages for the selective anticancer drug delivery and internal radiation.

In this study, the intracytoplasmic accumulation of Lipiodol in a proportion of HCC cells noticed within a short period of culture. The events that should happen in the cell membranes during the uptake of Lipiodol can not be explained by this experiment alone. If the intracytoplasmic accumulation of Lipiodol occurs in the HCC cells in vivo as in vitro, a long-lasting and selective therapeutic effect can be achieved by chemotherapy and internal radiation. The differences in Lipiodol uptake between the various types of benign and malignant cells remain to be answered.

In conclusion, Lipiodol accumulates in the cytoplasm and on the cell membrane in vitro. This accumulation may play a beneficial role for the selective and prolonged retention of Lipiodol in solid tumors, although the retention can be ascribed to the high leakage from tumor vasculature and to the underdeveloped lymphatics. In addition, the radioiodinated Lipiodol also has the same properties of accumulation in the cytoplasm and on the cell membrane. These characteristics of Lipiodol and radioiodinated Lipiodol will offer a long-lasting and selective therapeutic effect in chemotherapy and internal radiation.

REFERENCES

- Bookstein J: Hepatocellular carcinoma: Recent advances in diagnosis with iodized oil. *Radiol* 54: 253-254, 1985
- Fuchushima S, Kawaguchi T, Nishida M: Selective anticancer effects on 3', 5'-Diocanoyl-5 fluoro-2'-deoxyuridine, a lipophilic prodrug of 5-Fluoro-2'-deoxyuridine, dissolved in oily lymphographic agent on hepatic cancer of rabbit bearing VX-2 tumor. *Cancer Res* 47: 1930-1934, 1987
- Iwai K, Maeda H, Konno T: Use of oily contrast medium for selective drug targeting to tumor: Enhanced therapeutic effect on X-ray image. *Cancer Res* 44: 2115-2121, 1984
- Kanematsu T, Inokuchi K, Sugibayashi K, Kono K: Selective effects of lipiodolized anticancer agents. *J Sug Oncol* 25: 218-226, 1984
- Konno T, Maeda H, Iwai K, Maki S, Tashiro S, Uchida M, Miyauchi Y: Selective targeting of anticancer drug and simultaneous image enhancement in solid tumors by arterially administered lipid contrast medium. *Cancer* 54: 2367-2374, 1984
- Konno T, Maeda H, Iwai K, Tashiro S, Morinaga T, Mochinaga M: Effect of arterial administration of high molecular weight anticancer agent SMANCS with lipid lymphographic agent on hepatoma: A preliminary report. *Eur J Cancer Clin Oncol* 19: 1053-1065, 1983
- Nakakuma K, Tashiro S, Hiraoka T, Ogata K, Ootsuka K: Hepatocellular carcinoma and metastatic cancer detected by iodized oil. *Radiol* 154:15-18, 1985
- Ohishi H, Uchida H, Yoshimura H, Ohue S, Ueda J, Katsuragi M, Matsuo M, Hosogi Y: Hepatocellular carcinoma detected by iodized oil: Use of anticancer agents. *Radiol* 154: 25-29, 1985
- Ohnishi K, Tsuchiya S, Nakayama T, Hiyama Y, Iwama S, Goto N, Takashi M, Ohtsuki T, Kono K, Nakajima Y, Okuda K: Arterial chemoembolization of hepatocellular carcinoma with mitomycin C microcapsules. *Radiol* 152: 15-55, 1984
- Okayasu I, Hatekeyama S, Yoshida T, Yoshimatsu S, Tsuruts K, Miyamoto H, Kimura Y: Selective and persistent deposition of iodized oil, Lipiodol in the hepatocellular carcinoma after injection into the feeding hepatic artery. *Am J Clin Pathol* 90: 536-544, 1988
- Park C, Choi SI, Kim H, Yoo HS, Lee YB: Distribution of Lipiodol in hepatocellular carcinoma. *Liver* 10: 72-78, 1990
- Yoo HS, Suh JS, Lee JT, Kim BS, Choi HJ: Therapeutic trial of intrahepatic I-131-Lipiodol on patients with hepatocellular carcinoma. *The Kor J Gastroenterol* 18: 217-223, 1986

The Effect of Lipiodol on Human HCC Cell Line

Yoo HS, Park CH, Suh JH, Lee JT, Kim DI, Kim BS, Choi HJ, Madsen MT: Hepatocellular carcinoma: Treatment with a radiiodinated fatty acid ester. *Yonsei Med J* 29: 166-175, 1988

Yumoto Y, Jinno K, Tokujama K, Araki Y, Ishimitsu T, Maeda H, Konno T, Iwamoto S, Ohnishi K, Okuda K: Hepatocellular carcinoma detected by iodized oil. *Radiol* 154: 19-24, 1985
