Release Characteristics of a Free Polyunsaturated Fatty Acid from an Oily Lymphographic Agent

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The release profiles of a free polyunsaturated fatty acid, α-linolenic acid, from solutions in an oily lymphographic agent Lipiodol-Ultra-Fluid® (Lipiodol), to rabbit and human plasma, phosphate buffer solution (PBS), and PBS containing bovine serum albumin (BSA) were examined in vitro. The times required for 50% release of α-linolenic acid from Lipiodol were about 1 and 1.5 h in the rabbit and human plasma, respectively. Although only a slight amount of α-linolenic acid was released from Lipiodol to PBS after 24 h incubation at 37°C, release was markedly enhanced by the addition of BSA to PBS. The amount of α-linolenic acid released from Lipiodol into PBS containing 5% BSA increased as the α-linolenic acid content in Lipiodol was increased. In all experiments, the release had stopped before all α-linolenic acid had been released. The prolongation of α-linolenic acid release from Lipiodol is considered a requisite for a selective anticancer effect of Lipiodol containing a free fatty acid on liver cancer.

Keywords  free polyunsaturated fatty acid; α-linolenic acid; release; Lipiodol; albumin; in vitro

Introduction

The cytotoxicity of free polyunsaturated fatty acids against tumor cells has been reported.1,2 In our previous study,3,4 we observed the anti-hepatic-cancer effect of free fatty acids, i.e. linoleic acid, α-linolenic acid and γ-linolenic acid, dissolved in an oily lymphographic agent, Lipiodol-Ultra-Fluid® (Lipiodol), after administration into the hepatic artery using a rabbit liver cancer model.

The intra-hepatic-arterial administration of Lipiodol containing an anticancer drug is one of the most clinically effective chemotherapy agents against liver cancer.4–6 Since release patterns of an anticancer drug from Lipiodol formulations are expected to strongly influence its anticancer effect, several investigators have reported the drug release characteristics from Lipiodol formulations.7–10 In this study, we examined the release profiles of α-linolenic acid from Lipiodol, and the factor affecting the release profiles in vitro.

Experimental

Materials  α-Linolenic acid and pentadecanecoic acid were purchased from Sigma Chemical Co., and were more than 99% pure. Lipiodol was a product of Laboratories Guerbert. Bovine serum albumin (BSA), fraction V without essential fatty acids, was purchased from Sigma Chemical Co. All other chemicals were of reagent grade and obtained commercially. The rabbit plasma was obtained from a male New Zealand white rabbit and a freshly obtained plasma was used for experiments. The human plasma was obtained from a healthy volunteer and a freshly obtained plasma was used for experiments.

Preparation of α-Linolenic Acid Solutions in Lipiodol  α-Linolenic acid is liquid at room temperature and easily miscible with Lipiodol at any proportion. Therefore, the two substances were mixed with a vibrator mixer to prepare solutions with a desired α-linolenic acid content. α-Linolenic acid content in Lipiodol was 5 mg/100 μl except in the study on the effect of α-linolenic acid concentration in Lipiodol on its release.

Release Study  A solution of α-linolenic acid in Lipiodol (100 μl) was introduced into a prewarmed (37°C) release medium (10 ml) in a 30 ml volume Erlenmeyer flask and the content was shaken at 37°C. The α-linolenic acid solution sank and remained as a single droplet during the experiment. The release medium was human plasma, rabbit plasma, and 0.1 n phosphate buffer solution (PBS) at pH 7.4 with or without BSA. Rabbit plasma was selected since the anticancer effect of α-linolenic acid solution in Lipiodol after administration into the hepatic artery had been examined using a rabbit liver cancer model. Human plasma was selected since the data of release of α-linolenic acid in human plasma were considered to be important for the clinical application of α-linolenic acid solution in Lipiodol. A sample (300 μl) of the release medium was withdrawn at appropriate intervals and kept at −40°C until fatty acid was analyzed.

Fatty Acid Analysis  α-Linolenic acid concentrations in the release medium were measured by gas chromatography-mass spectrometry (GC-MS) (DX-303 DA 5000 system, JEOL). Samples (300 μl) of the release medium were added to 1 N HCl (100 μl), and α-linolenic acid in these mixtures was extracted with chloroform (3 ml) containing pentadecanoic acid as an internal standard and butylated hydroxytoluene (0.02%) as an antioxidant. After centrifugation (1500 g, 20 min at 4°C), the chloroform layer was collected and evaporated under reduced pressure. The residue was dissolved in ethanol, a solution of trimethylsilylimid hydroxide (MethyElute®, Pierce) intended for methylation was added and the mixture was introduced to GC-MS. The methylation was performed on a column according to the method of Middleditch and Desiderio.11 GC-MS conditions were as follows. The coil glass column (1.0 m × 3 mm i.d.) of the gas chromatograph was packed with 5% DEGS-H5PO4 on Chromosorb W (60–80 mesh). The injector, separator, and inlet temperature were 300, 250, and 260°C, respectively. The column temperature was raised from 200 to 216°C during a 1 min period. The carrier gas was helium and the flow rate was 40 ml/min. The electron impact mode at 70 eV was employed for the ionization of α-linolenic acid methyl ester and pentadecanoic acid methyl ester. Selected ion monitoring, m/z 292 for α-linolenic acid methyl ester and m/z 256 for pentadecanoic acid methyl ester, was performed.

Results

α-Linolenic Acid Release from Lipiodol to Humanand Rabbit Plasma  The α-linolenic acid release profiles from Lipiodol to human and rabbit plasma are shown in Fig. 1. The times required for 50% release were about 1 h in the rabbit plasma, and 1.5 h in the human plasma, and release has almost stopped within 3 h before all the acid had been released.

Effect of Albumin on α-Linolenic Acid Release  The α-linolenic acid release profiles from Lipiodol to PBS and

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Fig. 1. α-Linolenic Acid Release Profiles from Lipiodol into the Rabbit (O---O), and the Human Plasma (Δ---Δ) at 37°C α-Linolenic acid, 5 mg/100 μl. Averages of three experiments and bars indicate SE.
cytotoxicity of free fatty acids against tumor cells *in vitro*\(^{12-15}\) few studies have demonstrated the antitumor effect of these acids *in vivo*\(^{16,17}\). Furthermore, the only reported method of administration of free fatty acid has been with an emulsion intraperitoneally.\(^{16,17}\) One reason for the limited number of administration methods is the oily nature of free fatty acids.

Taking advantage of this oily nature, we have utilized them, linoleic acid, \(\gamma\)-linolenic acid and \(\alpha\)-linolenic acid in an oily formulation for a new concept of liver cancer chemotherapy: intra-hepatic-arterial injection of Lipiodol containing an anticancer drug.\(^{18,19}\) We observed significant suppression of the growth of cancer cells and a cytotoxic effect of these free fatty acid solutions in Lipiodol on liver cancer following their administration into the hepatic artery using the rabbit liver cancer model.\(^9\) In this chemotherapy, the characteristic of drug release from Lipiodol is one of the important factors in the antitumor activity.\(^7-10\) We therefore studied the characteristic of free fatty acid (\(\alpha\)-linolenic acid) release from Lipiodol *in vitro*. Although this release from Lipiodol *in vitro* might partially reflect the release profiles *in vivo*, there are several other possible factors affecting the latter: the droplet size of Lipiodol after administration into the hepatic artery; the state of distribution of Lipiodol in the blood vessels; the blood flow; the leakage of Lipiodol out of the blood vessels; the interaction between Lipiodol and cells; *etc.* The release profiles *in vivo* must be examined in a further study.

Little \(\alpha\)-linolenic acid release was observed when the release medium was PBS without BSA, because \(\alpha\)-linolenic acid is not soluble in PBS. In contrast, over 50% of the acid was released into rabbit and human plasma at 24 h. These results suggest the existence of a substance in plasma which enhances the \(\alpha\)-linolenic acid release.

We consider serum albumin to be one of the enhancing substances, because it binds free fatty acids with very high affinity and makes them soluble in plasma.\(^{20}\) The \(\alpha\)-linolenic acid release from Lipiodol was enhanced by the addition of BSA to PBS, and the amount released increased as the BSA concentration in PBS was increased. The amount released in human and rabbit plasma was almost equal to that released in PBS containing 3%—5% BSA, corresponding to the serum albumin content in plasma. The quantity of \(\alpha\)-linolenic acid in Lipiodol also affected its release, and the amount released increased as the quantity of \(\alpha\)-linolenic acid in Lipiodol was increased. The reason why the \(\alpha\)-linolenic acid release from Lipiodol was stopped before the amount released reached 100% (Fig. 1) and the percent of \(\alpha\)-linolenic acid released was decreased as the content of \(\alpha\)-linolenic acid in Lipiodol was increased (Fig. 2) is most likely that the solubility of \(\alpha\)-linolenic acid in the release medium almost reached maximum.

The \(\alpha\)-linolenic acid release from Lipiodol observed in the present study was higher than those reported for other Lipiodol formulations.\(^7-10\) The selectivity of intra-hepatic-arterial administration of Lipiodol consists of two factors: one is the selective blood supply to hepatic cancer only from the hepatic artery,\(^21\) and the other is selective disposition of Lipiodol in the hepatic cancer area after administration into the hepatic artery.\(^{18,22}\) If the drug release from Lipiodol was completed before the selective disposition of Lipiodol in cancer was achieved, the Lipiodol formulation

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**Fig. 2.** Effect of Albumin on the Profiles of \(\alpha\)-Linolenic Acid Release from Lipiodol into PBS without or with the Three Levels of BSA at 37°C
\(\alpha\)-Linolenic acid, 5 mg/100 μl. Averages of three experiments and bars indicate SE. ○ →, without BSA; □ →, 1% BSA; △ →, 5% BSA; ○ →, 5% BSA.

**Fig. 3.** Effect of \(\alpha\)-Linolenic Acid Concentration in Lipiodol on Its Release to PBS Containing 5% BSA at 37°C
Averages of three experiments and bars indicate SE. ○ →, 5 mg/100 μl; □ →, 10 mg/100 μl; ○ →, 20 mg/100 μl.

to PBS containing three amounts of BSA are shown in Fig. 2. Little release was observed from Lipiodol to PBS without BSA (release percent was only 0.3% at 24 h). Addition of BSA to PBS enhanced \(\alpha\)-linolenic acid release from Lipiodol and the percent of release increased as the concentration of BSA was increased. In each experiment, release had almost stopped within 3 or 7 h while some \(\alpha\)-linolenic acid still remained, and the release percent at 24 h was 29%, 60%, and 99% at BSA concentrations of 1%, 3% and 5%, respectively.

**Effect of \(\alpha\)-Linolenic Acid Concentration in Lipiodol on Release** The release profiles from Lipiodol containing three levels of \(\alpha\)-linolenic acid to PBS containing 5% BSA are shown in Fig. 3. The amount of \(\alpha\)-linolenic acid released during a certain period increased as its concentration in Lipiodol was increased. The amounts released at 24 h were 4.9 ± 0.5, 6.7 ± 0.7 and 9.6 ± 0.4 mg (mean ± S.E.) at the \(\alpha\)-linolenic acid concentration of 5, 10 and 20 mg/100 μl, respectively. Percent of \(\alpha\)-linolenic acid released during a certain period decreased as its concentration in Lipiodol was increased.

**Discussion**

Although there have been many reports on the
might take advantage of only the selective blood supply of the hepatic artery to cancer. Since ω-linolenic acid release from Lipiodol was almost completed within 3 h, it might be suggested that intra-hepatic-arterial administration of Lipiodol containing ω-linolenic acid took advantage of selective blood supply to hepatic cancer and took little advantage of selective disposition of Lipiodol.

Control of the release of fatty acids from Lipiodol is required in order to take advantage of this agent’s selective disposition. Our other data revealed that cell killing action of free polyunsaturated fatty acids is a product of concentration–time (area under the blood concentration–time curve (AUC) dependence). Therefore, prolongation of the ω-linolenic acid release from Lipiodol might be an effective approach, and this approach is now being examined in our laboratory.

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References