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The alteration of plasma lipoproteins by cremophor EL

Kathryn Woodburn and David Kessel

Department of Pharmacology, Wayne State University School of Medicine, Detroit, MI 48201 (USA)

Abstract

Cremophor EL (CRM) is a non-ionic amphipathic emulsifier which has been used as a parenteral vehicle for many hydrophobic drugs. At concentrations above 0.4 mg ml^{-1}, CRM alters lipoproteins so as to reduce their electrophoretic mobility; high density lipoprotein (HDL) is somewhat more sensitive than low density lipoprotein (LDL) in this regard. The same phenomenon was observed with Triton X-100 and Tween-80, two other non-ionic detergents. Ultracentrifugation experiments show that CRM alters the density of HDL resulting in a product with approximately the same specific gravity as LDL. Administration of mesoporphyrin solubilized with CRM to the mouse resulted in a rapid clearance of porphyrin bound to this new HDL species. Such a phenomenon may account for reports which indicate that CRM promotes the distribution of photosensitizing agents to neoplastic tissues.

1. Introduction

Cremophor EL (CRM), a non-ionic polyoxy-ethylated castor oil surfactant of complex composition, has been used to solubilize hydrophobic drugs for intravenous administration. These include the immunosuppressive drug cyclosporin [1] and an anti-tumor agent, taxol [2]. Clinical use of CRM has been associated with anaphylactoid reactions, hyperlipidemia and abnormal electrophoretic lipoprotein patterns [3]. Lipoprotein abnormalities have also been observed [4] in patients receiving miconazole therapy which was caused by the CRM present in the formulation. Changes in electrophoretic lipoprotein behavior have been attributed to the inhibition of plasma lipolytic enzymes by CRM [5].

Tin etiopurpurin (SnET2) is one of the "second generation" photosensitizers, a relatively hydrophobic agent whose formulation was initially carried out with CRM [6]. It was reported that CRM promoted binding of SnET2 to plasma lipoproteins [7, 8]. This resulted in enhanced tumor localization, consistent with lipoprotein-mediated distribution to tissues with elevated levels of low density lipoprotein (LDL) receptors. A recent report described interactions between CRM and plasma lipoproteins [9]. This work is part of a continuing project to assess the role of lipoprotein alterations as a factor in the plasma biodistribution of photosensitizing agents. We also examined the effects of two other non-ionic detergents on plasma lipoproteins.

2. Materials and methods

2.1. Chemicals and biological supplies

CRM, Tween-80 (TW80) and Triton X-100 (TX100) were obtained from Sigma Chemical Co., St. Louis, MO. Cholesterol and protein concentrations (Lowry method) were measured using diagnostic kits provided by Sigma. Mesoporphyrin (MP) (Porphyrin Products, Logan, UT) was dissolved in 0.1 M NaOH (1 mg ml^{-1}) or solubilized in 20% CRM+6% propylene glycol in isotonic saline [7]. High density lipoprotein (HDL) and LDL were isolated from pooled plasma samples by sequential flotation [10] and their purity was assessed by gel electrophoresis. Biodistribution studies were carried out with C3H mice bearing the RIF tumor; additional pharmacokinetic studies were performed with ICR mice. Plasma obtained from both C3H and ICR mice displayed the same electrophoretic and buoyant density characteristics.

2.2. Plasma profiles

The protein and lipoprotein components of the plasma were separated by density-gradient ultracentrifugation using a modification of a previously published procedure [11]. Plasmas (250 μl) were incubated with MP in the dark at 37 °C for 30 min, and then brought to a volume of 750 μl and a density of 1.21 g ml^{-1} with solid KBr and isotonic saline. This preparation was layered over 750 μl of KBr (density, 1.27 g ml^{-1}) in polyallomer centrifuge tubes (Beckman Instruments Inc., Palo Alto, CA). The tubes were then filled with isotonic saline (total volume, 3.9 ml). Separation of protein and lipoprotein was carried out by centrifugation using the Beckman TLV-1 table top ultracentrifuge and a TLN rotor (9° from vertical orientation) for 60 min at 100 000 rev min^{-1} ($r_{av} = 254\,000 \times g$). Tubes were fractionated from the top; a total of 25 fractions were collected. The distribution of the porphyrin into LDL, HDL and albumin fractions was assessed by fluorescence (excitation, 400 nm; emission, 620 nm) after dilution of each fraction to a final volume of 3 ml with TX100 (10 mM).

2.3. Animals

Female C3H mice bearing the RIF tumor were used [12]. MP was solubilized with 20% CRM or 0.1 M NaOH as described above, and administered via tail-vein injection. Three hours after MP administration the animals were sacrificed; blood and tumor tissue were collected. Blood was anti-coagulated with ethylenediaminetetraacetic acid (EDTA), erythrocytes were removed by centrifugation, and the resulting plasma sample was diluted into 3 mM TX100 and analyzed for MP as described above. Tumor tissue levels of MP were assessed by homogenizing 100 mg portions in 1 ml of ScintiGest (Fisher Scientific Co). After 2–3 days at room temperature, a clear solution was obtained; this was diluted to 3 ml with methanol and the MP level was determined by fluorescence as described above.

2.4. Gel electrophoresis

Lipoprotein purity and the effects of CRM, TW80 and TX100 on human and mouse lipoproteins were assessed by gel electrophoresis, using Paragon lipoprotein agarose gels (Beckman Instrument Co., Fullerton, CA). After electrophoresis in 50 mM barbital buffer (pH 8.6), the gels were fixed in a methanol–acetic acid mixture and stained with Sudan black (lipoprotein) or Coomassie blue (protein). Varying concentrations of the non-ionic detergent TX100, TW80 and CRM were incubated with human plasma and isolated lipoproteins for

30 min, and the effects of this procedure on the gel patterns were assessed.

3. Results

MP was used as a probe for the detection of the protein and lipoprotein components of human and mouse sera. The *in vitro* effects of varying CRM concentrations on plasma protein profiles of normal human plasma are shown in Fig. 1(a). In a separate experiment, we determined that the ultracentrifugation of MP in CRM micelles results in the appearance of these particles in fraction 11 (not shown), so that any free micelles would have been detected easily. In all studies, the positions of albumin and LDL were unchanged. We observed a shift in HDL to a lower specific gravity as the ratio of CRM to plasma was increased. At the highest CRM concentration (4.0 mg ml^{-1} whole plasma), the HDL profile was markedly broadened.

The *in vitro* effect of CRM on the distribution of MP to mouse plasma fractions is shown in Fig. 1(b). Similar tracings were observed with 0.24 and 1.6 mg CRM ml^{-1} whole plasma concentrations; a CRM concentration of 4.0 mg ml^{-1} decreased the density of the MP-binding fractions, as was also observed with human plasma.

A time dependence study was performed in ICR mice to observe the "clearance" profile of the different lipoprotein fractions (Fig. 2(a)). CRM was injected into 20 g female mice (0.1 ml of 20%
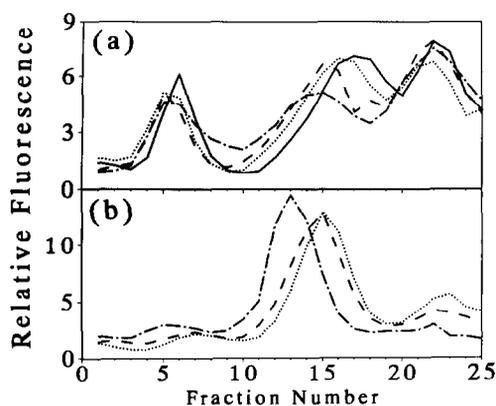

Fig. 1. The *in vitro* effect of increasing CRM concentrations on human (a) and mouse (b) plasma protein profiles: ——, control plasma; ···, 0.24 mg CRM ml^{-1} whole plasma; – – –, 1.6 mg CRM ml^{-1} whole plasma; – · –, 4 mg CRM ml^{-1} whole plasma. In (b) the 0.24 mg CRM ml^{-1} concentration exhibited the same profile as the control plasma. In human plasma, fractions 3–7 represent LDL, fractions 13–18 are HDL and fractions 20–25 are albumin. The degraded phospholipid fraction cannot be distinguished from LDL by density-gradient centrifugation.
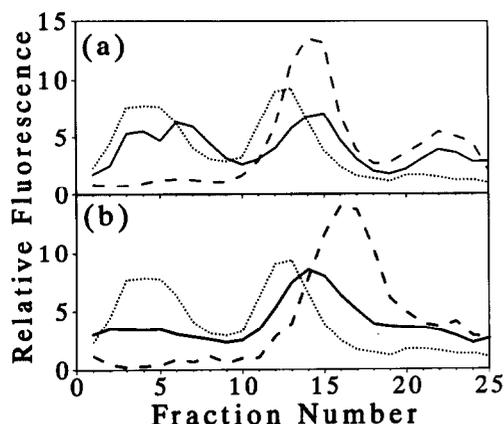
Fig. 2. The *in vivo* effect of CRM on mouse plasma proteins. (a) Time dependence of CRM on mouse plasma fractions: control and 24 h exhibited the same profile (broken line); 1 h (dotted line); 3 h (full line). (b) The effect of different solvents on mouse plasma fractions. Injections 1 h before sampling: 0.1 ml 20% CRM alone (dotted line) with the lipoproteins identified by the addition of MP after ultracentrifugation; 0.1 ml of MP in 20% CRM (full line); 0.1 ml of MP in 10 mM NaOH (broken line).

$CRM = 20$ mg mouse^{-1}), and the plasma samples were collected 1 and 3 h later. A 125 μl aliquot of each plasma sample was mixed with MP (final concentration, 16 μg ml^{-1}) and the lipoproteins were separated by ultracentrifugation. This experiment provided a measurement of the relative distribution of lipoproteins in the different fractions. Injection of CRM resulted in the appearance of a "pseudo-LDL fraction", with the mouse HDL appearing in a region normally occupied by LDL. The "masquerade" was over by 24 h with the same plasma profile as that of the control. This same effect was observed with C3H mice.

To determine the effect of CRM on the fate of MP *in vivo*, we injected MP (5 mg kg^{-1}) either dissolved in 10 mM NaOH or solubilized with 20% CRM (total volume of injection, 0.1 ml). Blood was collected 1 and 3 h after administration; the results are shown in Fig. 2(b). In both cases, we observed the distribution of the drug to a single broad fraction consistent with the density-gradient behavior of HDL.

Changes in the electrophoretic migration patterns of human plasma caused by CRM, TW80 and TX100 were examined after incubation of these products with human plasma *in vitro*. The results of incubating increasing detergent concentrations with whole human plasma was assessed (not shown). In all cases, a marked alteration in electrophoretic behavior was associated with the appearance of the more slowly migrating bands. The threshold concentrations (mg ml^{-1}) needed to produce an effect on the electrophoretic mobility

patterns of whole human plasma were 0.4 for TW80, 0.8 for CRM and 1.6 for TX100.

The effect of increasing CRM concentrations on isolated human HDL and LDL is shown in Fig. 3 (top) and that of TW80 in Fig. 3 (bottom). To draw a relative comparison of the effects of the detergent on the lipoproteins, equivalent HDL and LDL protein concentrations (0.5 mg ml^{-1}) were used. The minimum TW80 and CRM level needed to produce an effect on isolated HDL and LDL was 0.4 mg ml^{-1} (*i.e.* 0.33 mg detergent mg^{-1} protein). HDL was more sensitive to the detergent interaction than LDL. TW80 had a marginally (compare lanes 2 and 6 in each of the gels in Fig. 3) greater effect on the electrophoretic mobility of the lipoproteins than CRM.

It should be noted that density-gradient centrifugation cannot distinguish between LDL and
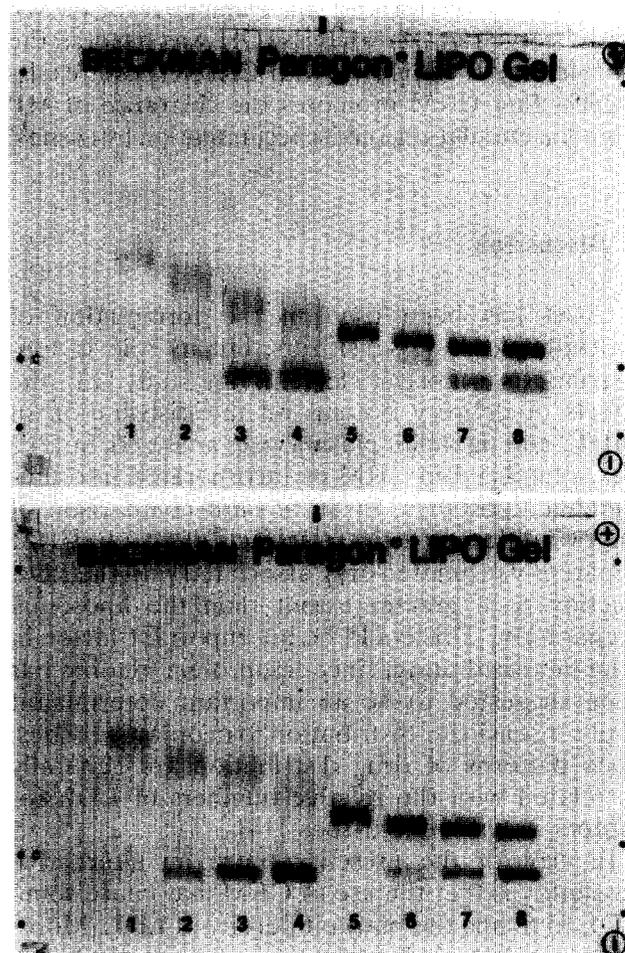

Fig. 3. Electrophoretic changes induced by CRM (top) and TW80 (bottom) on isolated human HDL and LDL. Lane 1 is normal HDL; lanes 2–4, incubation of HDL with increasing detergent concentrations (0.4, 0.8 and 1.6 mg ml^{-1}); lane 5 is normal LDL; lanes 6–8, incubation of LDL with increasing detergent concentrations (0.4, 0.8 and 1.6 mg ml^{-1}).

TABLE 1. Distribution of mesoporphyrin to tumor and plasma

Vehicle	Tumor (μg g^{-1})	Plasma (μg ml^{-1})
NaOH	2.35 ± 0.07	0.341 ± 0.035
CRM	2.77 ± 0.29	0.293 ± 0.009

Plasma and tumor samples were obtained 3 h after administration of MP (5 mg kg^{-1}) to tumor-bearing mice. Results reflect data obtained from three separate experiments.

the released phospholipid fraction, although the latter can readily be delineated by gel electrophoresis (Fig. 3). Using the latter technique, we detected the presence of the phospholipid in fractions 2–7 of human plasma after exposure to CRM (not shown).

The biodistribution of MP to the RIF tumor in vivo showed that formulation with CRM enhanced drug accumulation (Table 1). Studies on the plasma levels of MP indicated that a higher level of MP was observed at 3 h when NaOH was used as the delivery agent compared with CRM. These results suggest that CRM promotes the clearance of MP from the circulation and its accumulation by tissues.

4. Discussion

CRM has been used in the formulation of hydrophobic photosensitizers, but its role in biodistribution is not yet clear. In one study [13], the effects of delivery systems on the distribution of SnET2 among rabbit plasma were analyzed. SnET2 was solubilized in CRM or incorporated into unilamellar liposomes. In an in vitro comparison of the two different delivery systems after 2 h, CRM apparently promoted drug associated with the LDL fraction to a greater extent than the liposomal preparation. This could be an important factor in drug biodistribution, since lipoprotein binding has been suggested to be an important determinant of photosensitizer distribution to neoplastic tissues, since patterns of drug distribution are generally correlated with the relative numbers of LDL receptors in different tissues [14].

In view of the present report, an alternative possibility is that the use of CRM may cause some HDL to mimic the buoyant density of LDL. Moreover, a CRM-induced lipoprotein degradation product could be detected by gel electrophoresis which may also play a role in drug biodistribution.

Kongshaug et al. [9] examined the interaction of CRM with human plasma in vitro, using ultracentrifugation to separate lipoprotein species.

They reported a CRM-induced change in HDL, resulting in a decrease in the density of the particles, on addition of 1–4 mg of CRM per milliliter of plasma. As the CRM level increased, the HDL particles disappeared, and species of both higher and lower densities were observed. We did not observe the CRM-induced formation of "heavy" human HDL fractions described by Kongshaug et al. [9], but found a conversion of HDL to a lower density species. The effect was observed in both in vitro and in vivo environments, but was more pronounced in vivo. Both the density and electrophoretic migration of HDL changed with increasing detergent concentrations. While the electrophoretic mobility of LDL changed markedly, there was no change in buoyant density detectable by density-gradient ultracentrifugation.

There are few reported studies on the effects of CRM on plasma lipoproteins. Hacker et al. [15] examined the CRM-induced alteration of serum lipids in dogs: electrophoresis of sera revealed a decreased α-lipoprotein fraction and the appearance of an unidentified peak near the origin in the chylomicron region. We found that the lipoprotein degradation product(s) stain with Sudan black but not with Coomassie blue, suggesting that they represent lipid-rich phospholipid fragments. While MP is a poor tumor localizer, it is a very good fluorescent probe for plasma lipoproteins and was utilized for this purpose.

It is interesting to note that MP, bound to the phospholipid fraction (tubes 2–6, Fig. 2(b)), is rapidly cleared from the circulation (Fig. 2(a)). This result may offer an explanation for the decreased plasma and enhanced tumor levels of MP found when CRM is used as the drug vehicle (Table 1). In other studies, to be reported elsewhere, we found that the complex between this degraded lipoprotein component and other photosensitizers is not rapidly cleared from plasma. The role of the phospholipids released from HDL and LDL in sensitizer biodistribution remains to be explored.

We interpret these results to indicate that CRM alters the physical characteristics of HDL and LDL. The density of HDL is changed to a product with the same specific gravity as LDL so that, in the absence of studies involving gel electrophoresis, it might be concluded that the promotion of sensitizer binding to LDL had occurred. Moreover, exposure to CRM results in the release of a phospholipid from both HDL and LDL. These phenomena are more pronounced in vivo than in vitro. In view of the present work, together with Kongshaug et al.'s report [9], caution is needed

with regard to the interpretation of studies on the distribution of sensitizers to different lipoprotein species in the presence of CRM and other detergent solubilizers.

Acknowledgments

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