The exogenous surfactant Curosurf enhances phosphatidylcholine content in isolated type II cells

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ABSTRACT: The exogenous surfactant, Curosurf, contains proteins as well as phospholipids. We investigated the possibility that these might affect the reutilization of exogenous phospholipid by type II alveolar cells isolated from rat lung.

The time course of incorporation into lamellar bodies of radioactivity from triitated dipalmitoyl-phosphatidylcholine (DPPC) contained within liposomes was studied. Rates of uptake were compared between liposomes prepared from pure phospholipids and from Curosurf. The incorporation of labelled choline into newly synthesized phosphatidylcholine was also determined in the presence of both preparations.

The rate of DPPC incorporation over the first 4 h was the same, but, after 6 and 8 h, the radioactivity associated with lamellar bodies was about 40% higher from Curosurf liposomes. By contrast, both Curosurf and the phospholipid mixture enhanced choline incorporation into phosphatidylcholine to the same extent.

We conclude that Curosurf enhances the surfactant-related phosphatidylcholine content of type II cells by two mechanisms, one of which depends on the presence of proteins in exogenous surfactant. The difference in incorporation of radioactivity from liposome-associated labelled dipalmitoyl-phosphatidylcholine cannot be explained just by an increase in reutilization of choline from degraded dipalmitoyl-phosphatidylcholine.


The endotracheal instillation of exogenous surfactant has been shown to improve lung function in premature newborns with surfactant deficiency. Surfactant replacement therapy has, thus, recently become a routine treatment of respiratory distress syndrome (RDS). Pulmonary surfactant is a phospholipid-rich material, of which the major component is phosphatidylcholine (PC). Curosurf is a modified natural surfactant isolated from pig lung [1]. Its efficiency in vivo has been demonstrated experimentally [2], and the benefit of its use in the treatment of RDS has been established by multicentre clinical studies [3, 4]. In addition to this immediate physiological effect, Curosurf may also affect the biology of alveolar type II cells that elaborate endogenous surfactant. Curosurf is characterized by the presence in the surfactant lipid extract, of the hydrophobic, phospholipid-associated surfactant proteins (SP) B and C. Since, on the one hand, the major clearance pathway of alveolar surfactant is reuptake by type II cells with subsequent reutilization [5, 6], and on the other hand, SP-B and SP-C have been shown to be involved in this process of surfactant reuptake [7–9], their presence may favour reutilization of instilled surfactant material, as compared with artificial surfactants which do not contain these proteins.

A first part of the present study, therefore, aimed at testing this hypothesis. Moreover, exogenous surfactants are administered to premature newborns, whose lungs are rapidly maturing. This fast maturation process, with increasing production of endogenous surfactant, is crucial for the recovery from RDS. Another aspect of the study was, therefore, to determine whether or not the presence of Curosurf in contact with type II cells affects the rate of synthesis of endogenous surfactant. The isolated cultured type II cell is an adequate model for these investigations. This model makes it possible to follow the time course of surfactant reuptake and incorporation of material into lamellar bodies, and has, indeed, been used for studying factors that modulate surfactant reuptake [5, 10]. It is also suitable for measuring the rate of endogenous surfactant synthesis [11], and to evaluate the influence of various factors on the maturational process [12].

Material and methods

Animals

Virgin Wistar female rats (Charles River, St Aubin-lès-Elbeuf, France) were mated overnight in the laboratory. The following morning was designated day 0 of gestation. Pregnancy was checked by palpation 14 days later. Type II cells were isolated from the lungs of 19 and 20 day old rat foetuses. Gestational day 20 in the
rat (term 22 days) corresponds to a developmental stage when type II cells are fully differentiated with regard to their ability to elaborate all surfactant components, although they are not fully mature [13]. This stage was elicited for phospholipid uptake and reutilization studies. Day 19 corresponds to the stage when surfactant storage starts [13]; it has been chosen for evaluating the potential effects of Curosurf on the rate of endogenous surfactant synthesis. Uteline horns were removed en bloc under pentobarbital anaesthesia and transferred to the laminar-flow hood where foetuses were extracted and foetal lungs removed under aseptic conditions.

**Cell cultures**

Type II cells were isolated according to Bates and colleagues [14], with slight modifications. In brief, chopped foetal lung tissue was submitted to four successive enzymic dissociation steps, 20 min each, in Eagle's minimum essential medium (MEM; Gibco-BRL, Eragny, France) supplemented with trypsin 0.1%, collagenase 0.1% and deoxyribonuclease (DNase) 0.002%. At each step, when the remaining tissue had been decanted, dissociated cells were taken up with medium and fresh enzymic solution was introduced. Enzyme-containing medium was removed by centrifugation, and MEM containing 10% foetal bovine serum (FBS; DAP, Vogelgrun, France) was added to the cells. The cell suspensions collected were filtered sequentially on 48, 25 and 15 µm nylon mesh (Nitex) to mechanically separate the cells. Cells were counted and adjusted to 1.7×10⁶ cells·mL⁻¹. Three successive adhesion steps on plastic culture dishes, 45 min each, removed most fibroblastic cells. Furthermore removal of remnant fibroblasts was achieved by four centrifugations in MEM, each 3 min at 12000 g [15]. The purity of the resulting epithelial cell population was evaluated by labelling for vimentin and cytokeratin [16]. The purity of the resulting epithelial cell population was evaluated by labelling for vimentin and cytokeratin [16]. The preparations were found to be 99 and 86% epithelial for cells isolated at 19 and 20 days, respectively (2,000 cells sample⁻¹). All preparations were found to be 99 and 86% epithelial for cells isolated at 19 and 20 days, respectively (2,000 cells sample⁻¹).

**Preparation of liposomes**

Phospholipid vesicles prepared by sonication of phospholipid suspension in aqueous solution (liposomes) are classically used for uptake studies. Liposomal PC is taken up intact and incorporated into subcellular organelles, including lamellar bodies [17]. We referred to the study by Bates et al. [18] for the final concentration of total PC in the culture medium. Due to the difficulty of total removal of proteins from Curosurf to obtain a phospholipid fraction, a mixture of pure phospholipids was prepared and used as a protein-free surfactant-like reference compound. Phospholipids were purchased from Sigma. A phospholipid mixture was prepared with composition similar to that of natural porcine surfactant [19], and with the same concentrations of total phospholipids and PC as Curosurf. The phospholipid concentration of Curosurf is 80 mg·mL⁻¹, of which 75% is PC (Communication from Serono labs).

Composition of the mixture made for preparing phospholipid liposomes is presented in table 1. It was prepared from stock solutions of individual phospholipids in chloroform/methanol 2:1 (v:v). The mixture was dried under sterile nitrogen flux and redissolved by stirring in an appropriate amount of sterile phosphate buffered saline (PBS). For Curosurf, 12.5 µL of the suspension was diluted in PBS up to 1 mL. Liposomes were prepared by sonication of both suspensions for 30 s·min⁻¹ over a period of 15 min, in ice [20]. For precursor incorporation studies, liposomes were unlabelled. For the DPPC reutilization study, they were labelled before sonication with 1,3-dipalmitoyl-(N methyl ³H) choline, dipalmitoyl [³H]-DPPC 80 Ci·mmol⁻¹ (2.96 TBq·mmol⁻¹, Amersham-France, Les Ulis, France). [³H]-DPPC, 20.8 µCi (770 kBq), were added per millilitre of phospholipid and Curosurf suspensions. To 1 mL of DM was added 24 µL of either phospholipid or Curosurf liposome suspension. The final concentrations were about 24 µg·mL⁻¹ DM of total phospholipids and 18.5 µg·mL⁻¹ DM of total phosphatidylcholine. For radiolabelled liposomes, the activity was 0.5 µCi (18.5 kBq)·mL⁻¹ and the specific activity of [³H]-DPPC was 27 µCi (1 MBq)·mg⁻¹ phosphatidylcholine.

**Table 1.** Composition of phospholipid liposomes

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>%</th>
<th>µg·mL⁻¹ suspension¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>45</td>
<td>475</td>
</tr>
<tr>
<td>Egg lecithin</td>
<td>30</td>
<td>313</td>
</tr>
<tr>
<td>PG</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>PE</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Sm</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>PI</td>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>PS</td>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td>lyso PC</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

¹: final concentration. DPPC: dipalmitoyl-phosphatidylcholine; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; Sm: sphingomyelin; PI: phosphatidylinositol; PS: phosphatidylserine; lyso PC: lysophosphatidylcholine.
**DPPC uptake study**

Type II cells from 20 day foetal lungs were continuously exposed to DM containing radiolabelled liposomes (2.5 mL·well⁻¹) for times ranging 1–10 h. Determinations were made for all time-points of the study in each experiment. At the end of the contact period, DM was removed, and cells were rinsed with Tris-ethylenediamine tetra-acetic acid (EDTA) NaCl (TEN) buffer (10, 1 and 154 mM, respectively), pH 7.4, then scraped with the aid of a rubber policeman in the same buffer, and kept at -20°C until further processing.

**Incorporation of [³H]-choline and [³H]-acetate**

For evaluating the influence of phospholipid mixture and Curosurf on the rate of incorporation of [³H]-choline and [³H]-acetate into endogenously formed phospholipids, either 0.1 µCi (3.7 kBq) of [methyl ³H]-choline (80.7 Ci·mmol⁻¹ [3.03 TBq·mmol⁻¹]; Amersham-France) or 2 µCi·mL⁻¹ (74 kBq) of [³H]-acetate (2.18 Ci·mmol⁻¹ [80.7 GBq·mmol⁻¹]; Amersham-France) were added per millilitre of DM [12]. One or the other labelled precursor and liposomes were simultaneously introduced to the medium of cultured 19 day cells. Incorporation was allowed to occur for 24 h. Cells were then rinsed, scraped and kept frozen in TEN buffer as above.

**Extraction of lamellar bodies (surfactant fraction) from cultured cells**

In order to determine whether DPPC taken up from the medium is reutilized for surfactant material destined for secretion, or if phospholipids synthesized from radio-labelled precursors are destined for the surfactant compartment, it is necessary to determine whether radioactivity is integrated into lamellar bodies that must, therefore, be extracted from cultured cells. The technique of extraction used was derived from the one initially described by FrisoloLo et al. [21], and adapted for extraction from cultured cells and quantitative analysis [11]. The fraction isolated by this method has been shown to select lamellar bodies and to exhibit all the characteristic biochemical and biophysical features of surfactant [22]. In brief, homogenates in TEN buffer were deposited on sucrose 0.75 M in TEN buffer and centrifuged for 1 h at 48,000×g. Material at the interface between sucrose and supernatant was collected, diluted with TEN, deposited on a discontinuous sucrose gradient 0.25 and 0.68 M sucrose in TEN, and centrifuged for 1 h at 64,000×g. The final surfactant fraction banded at the interface between the two sucrose concentrations. The so-called residual fraction (nonsurfactant fraction) gathering pellets and supernatants of the two centrifugation steps was discarded in DPPC reutilization experiments, but collected and analysed in [³H]-choline incorporation experiments.

**Determination of radioactivity of reuptaken DPPC**

Lipids from surfactant fractions were extracted by vigorous shaking with chloroform/methanol/water, 1:2:0.8 (v:v:v) and collected in the lower organic (chloroform) phase. After drying-up of the phase, lipids were redissolved with 0.5 mL of chloroform/methanol, 2:1 (v:v), and an aliquot fraction was taken up for counting of radioactivity in Optiscint scintillation cocktail (EEG Instrument, Evry, France), with the aid of a LKB rack β-counter.

**Separation of phospholipids and determination of radioactivity in precursor incorporation experiments**

Lipids were extracted, as described above, from whole cells or from both surfactant and residual fractions isolated from cultured cells. Individual phospholipids were separated by one-dimensional thin layer chromatography on silica gel 60 chromatography plates (Merck, Darmstadt, Germany), in a solvent system chloroform/hexane/methanol/glacial acetic acid/water, 12:7:4:3:0:3 (v:v:v:v:v) [23]. In the instance of acetate incorporation, further extraction of disaturated phosphatidylcholine (DSPC) from total phosphatidylcholine (PC) was performed by the osmium tetroxide method and thin layer chromatography separation in chloroform/methanol/water, 65:25:4 (v:v:v), according to Patterson et al. [24]. Phospholipids were visualized with iodine, identified by comparison with standards run in parallel, scraped, eluted by chloroform/methanol/water 1:2:0:8 (v:v:v), dried, redissolved in a known volume of chloroform/methanol, 2:1 (v:v), and their activity counted in Optiscint, as described above.

**Deoxyribonucleic acid (DNA) assay**

In order to compare data between various culture experiments, radioactivity incorporated was normalized to cell DNA determined by a fluorometric method [25] using fluorescent dye, Hoechst 33258 (λ excitation: 350 nm; λ emission: 455 nm).

**Statistical analysis**

Data are presented as mean±SEM. Comparison of mean values was made by analysis of variance (ANOVA) and, when applicable, by t-test for paired values. A p-value of less than 0.05 was considered significant.

**Results**

**Uptake of DPPC from liposomes**

Incorporation of radioactivity from labelled DPPC into lamellar bodies was studied over 10 h by comparing liposomes prepared from Curosurf and from protein-free phospholipid liposomes. Ten separate experiments were performed. In each experiment, the results were essentially the same, despite slight variations in the time course profile. In all experiments, the initial rate of incorporation of radioactivity from [³H]-DPPC into lamellar bodies was almost identical, despite slight variations in the time course profile.
first 2 h of the study. At later times, by contrast, the incorporated radioactivity was always found to be higher in the case of Curosurf, although the time when the values became different varied from 4 to 8 h after initiation of contact. The average values of the whole study are depicted in figure 1. Statistical analysis for each time-point showed that the mean level of radioactivity incorporated was significantly higher for Curosurf (about 40%) at times 6 and 8 h. Differences at other time-points were not significant. The surface area between the two curves from times 4 to 10 h can be interpreted as the amount of additional stored material in the case of Curosurf as compared with the control condition.

**Incorporation of [3H]-choline**

Results are summarized in table 2. With regard to incorporation in whole cells, no difference was observed between either phospholipid or Curosurf liposomes and control DM, for radioactivity incorporated in total lipid extract as well as in PC. With regard to the surfactant fraction, both phospholipids and Curosurf significantly increased incorporation of the precursor into total lipid extract and PC. The increase for total lipid extract was 35 and 22% and that in PC 36 and 27 for phospholipid and Curosurf liposomes, respectively.

**Incorporation of [3H]-acetate**

Table 3 summarizes the results of acetate incorporation in various lipids of cultured cells. The distribution of radioactivity was consistent with previous observations [12]. Acetate incorporation into total PC was unchanged by phospholipid liposomes but significantly diminished (-23%; p<0.05) by Curosurf as compared with that in control DM. In the surfactant fraction, however, no significant change was induced by phospholipids (165±16 disintegrations per minute (dpm)-µg⁻¹ DNA) or by Curosurf (133±10 dpm-µg⁻¹ DNA), as compared with DM (159±14 dpm-µg⁻¹ DNA). The incorporation into DSPC was unchanged by phospholipids or by Curosurf, in whole cells as well as in surfactant. Neither phospholipids nor Curosurf significantly changed acetate incorporation into other phospholipids or neutral lipids.

**Discussion**

The present study was undertaken to determine whether contact with the exogenous porcine surfactant Curosurf may influence functions of the developing alveolar type II cell. The results indicate that Curosurf enhances reincorporation of external PC and synthesis of endogenous PC from choline in the surfactant compartment of isolated type II cells.

The time course of the incorporation into lamellar bodies of radioactivity from labelled-DPPC was compared between liposomes formed from Curosurf and

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Fig. 1. – Incorporation of radioactivity from labelled dipalmitoylphosphatidylcholine into lamellar bodies induced by Curosurf (—●—) or protein-free phospholipid liposomes (.....). Values are mean±SEM. cpm: counts per minute; DNA: deoxyribonucleic acid. *: p<0.05; **: p<0.01, compared to phospholipid liposomes.

Table 2. – Effects of phospholipid and Curosurf liposomes on [3H]-choline incorporation by isolated alveolar type II cells

<table>
<thead>
<tr>
<th></th>
<th>Whole cells TLE</th>
<th>PC</th>
<th>Surfactant fraction TLE</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DM</td>
<td>3943±242</td>
<td>2542±155</td>
<td>156±13</td>
<td>117±9</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>3707±257</td>
<td>2684±155</td>
<td>211±16*</td>
<td>160±12**</td>
</tr>
<tr>
<td>Curosurf</td>
<td>3385±227</td>
<td>2362±156</td>
<td>190±14*</td>
<td>149±11*</td>
</tr>
</tbody>
</table>

Values are presented as mean±SEM of 16 samples from three different culture experiments. Cells were isolated from 19 day foetal lung. Results are expressed as dpm·10⁻⁵ cells per 24 h. TLE: total lipid extract; PC: total phosphatidylcholine; DM: defined medium (for composition see text); dpm: disintegrations per minute. *: p<0.05; **: p<0.01, significant difference from corresponding control value (analysis of variance (ANOVA) and t-test).

Table 3. – Effects of phospholipid and Curosurf liposomes on [3H]-acetate incorporation by isolated alveolar type II cells

<table>
<thead>
<tr>
<th></th>
<th>PC</th>
<th>DSPC</th>
<th>Sm</th>
<th>PE</th>
<th>PS + PI</th>
<th>PG</th>
<th>Other lipids⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DM</td>
<td>2204±204</td>
<td>575±40</td>
<td>430±48</td>
<td>221±10</td>
<td>112±10</td>
<td>21.5±2.5</td>
<td>587±25</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>2499±289</td>
<td>534±28</td>
<td>384±29</td>
<td>195±18</td>
<td>94.5±11</td>
<td>19±2</td>
<td>506±34</td>
</tr>
<tr>
<td>Curosurf</td>
<td>1701±120*</td>
<td>515±67</td>
<td>332±49</td>
<td>205±12</td>
<td>116±8</td>
<td>26.5±3.5</td>
<td>628±23²</td>
</tr>
</tbody>
</table>

⁷: including neutral lipids. Values are presented a mean±SEM of eight samples per experiment (expressed as dpm-µg⁻¹ DNA). Cells were isolated from 19 day foetal lung. PC: phosphatidylcholine (total); DSPC: disaturated phosphatidylcholine; DNA: deoxyribonucleic acid. For further definitions see legends to tables 1 and 2. *: p<0.05 significant difference from corresponding control value (t-test for paired values); †: p<0.05 significant difference from corresponding phospholipid value (t-test for paired values).
those from an artificial phospholipid mixture. After an initial period of about 4 h, when the pattern of incorporation was the same for both preparations, Curosurf enhanced the incorporation rate as compared to liposomes devoid of proteins. The identity of initial uptake profiles indicates that, despite the fact that the phospholipid composition of both preparations was not strictly identical, the PC concentrations and DPPC specific activities were effectively the same.

The biphasic profile of the incorporation curves suggests that different mechanisms may be involved during the two different phases. The first phase, from time 0 to 2–4 h, may correspond essentially to recycling of intact exogenous DPPC into lamellar bodies. The second phase, beyond 4 h, may correspond to a more complex situation involving both recycling of intact DPPC and reincorporation (i.e., resynthesis) of material from degraded DPPC, a mechanism previously shown to take place in type II cells [6]. The stimulating effect of Curosurf on recycling of DPPC radioactivity becomes evident rather late, whereas, in previous experiments [7, 9], the stimulating effects of lipophilic surfactant proteins were observed immediately during the first hour. This may mean that Curosurf enhanced reutilization of components of degraded DPPC for resynthesis rather than uptake and recycling of intact DPPC. It should be emphasized, however, that in previous investigations, adult type II cells were used, whereas foetal cells were used in the present study. Thus, the late effect of Curosurf may also reflect a slower recycling process in immature cells, consistent with the longer half-life and turnover time of surfactant components in the lung of immature animals [10, 26].

Both liposomes prepared with the phospholipid mixture or with Curosurf stimulated the biosynthesis of surfactant phosphatidylcholine from choline by cultured lung type II cells. This may result from the presence in both preparations of phosphatidylglycerol, a surfactant component, with a stimulating effect on the rate of phosphatidylcholine synthesis that has been demonstrated previously, through activation of the rate-limiting enzyme of the pathway, choline phosphate cytidylyltransferase [27, 28].

Changes induced by liposomes in acetate incorporation into various lipids and phospholipids were very limited. Although a decrease was observed with Curosurf in total PC, it should be emphasized that incorporation into the most functionally important phospholipid, i.e., DSPC, was not diminished in whole cells; neither was that in PC of the surfactant fraction. The apparent decrease of acetate incorporation into PC of whole cells in the presence of Curosurf may reflect a dilution of labelled fatty acids by unlabelled fatty acids from Curosurf phospholipids. This assumption is supported by the absence of change for acetate incorporation into neutral lipids, suggesting that lipogenesis was not affected. From both precursor incorporation approaches, it can be concluded that neither Curosurf nor phospholipid liposomes influenced the entry of acetate into surfactant PC, but that they both stimulated the de novo PC synthetic pathway that leads to condensation of choline with the lipid precursor diacylglycerol [29].

The fact that both preparations stimulated choline incorporation into PC indicates that the increased incorporation of radioactivity from labelled DPPC induced by Curosurf after 6–8 h cannot be accounted for only by re-incorporation of labelled choline from degraded DPPC. If this had been the case, there would have been no significant difference between Curosurf and phospholipid liposomes. The differences observed in DPPC reutilization are likely to be due to the presence of hydrophobic surfactant proteins in Curosurf. SP-B has been shown to facilitate liposome uptake by nonsaturable, non-specific mechanisms [9]. Effects previously attributed to SP-C have been reported in one study [18] to be due, in fact, to phosphatidylglycerol and phosphatidylethanolamine co-isolated with the protein. However, a more recent study [8] clearly indicated that pure SP-C enhanced binding and endocytosis of lipid vesicles by type II cells or cells of the MLE-12 line, and was more potent than SP-B in this respect. In the present experiments, the difference in DPPC reutilization between phospholipid liposomes and Curosurf could, therefore, be accounted for both by SP-B and SP-C.

In summary, Curosurf appears to enhance the surfactant phosphatidylcholine content of type II cells by two different mechanisms. As a phosphatidylglycerol containing surfactant, it stimulates phosphatidylcholine synthesis. Because it contains hydrophobic surfactant proteins, it may also favour reutilization of surfactant phospholipids. As far as the present observations obtained with isolated cells could be extended to alveolar cells in vivo, Curosurf, in addition to its immediate positive effects on lung function in the premature newborn, would favour both reutilization of its phospholipid components and surfactant phosphatidylcholine synthesis from its precursors.

References


