Effects of clodronate-containing liposomes on testicular macrophages and Leydig cells in vitro

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Abstract

We undertook the present studies to determine if clodronate-containing liposomes have direct effects on Leydig cells. Macrophages and Leydig cells were isolated and maintained separately in culture. Following treatment with clodronate-containing liposomes, macrophages were killed in a dose–response fashion over a range of 5–200 µl liposomes. By comparison, a 500 µl dose was required to kill Leydig cells, but this was not dependent upon clodronate since liposomes containing buffer elicited an identical response. The concentration of testosterone in medium from Leydig cells treated with clodronate-containing liposomes was significantly reduced compared with untreated cells. However, we subsequently found that liposomes can adsorb testosterone. Therefore, testosterone production was determined at various times following removal of liposomes from Leydig cells, thereby circumventing this complication. It was found that testosterone production was not altered by liposomes under these conditions. Finally, free clodronate had no effect on testosterone production, even at doses representing the amount present within the 500 µl dose of liposomes. In summary, clodronate-containing liposomes killed testicular macrophages at a far smaller dose than required to kill Leydig cells. Most importantly, neither liposomes nor free clodronate had a direct effect on testosterone production. Thus, clodronate-containing liposomes represent a valuable tool to study Leydig cell–macrophage interactions.

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Introduction

While luteinizing hormone (LH) plays an important role in regulating testosterone production by Leydig cells it is clear that local factors are also involved. For example, it has been demonstrated that secreted products from cultured testicular macrophages alter testosterone production by cultured Leydig cells (for reviews see Hutson 1994, Hales 1996). Studies conducted in vitro also indicate that testicular macrophages are functionally coupled to Leydig cells. An early elegant study demonstrated that intratesticular injections of silica, a substance known to be cytotoxic to macrophages (Kagan & Hartmann 1984), reduced the level of serum testosterone (Huhtaniemi et al. 1986). More recently, it has been shown that testosterone secretion by Leydig cells is altered when macrophages are experimentally depleted from the testis using liposomes containing clodronate (dicloromethylene-bisphosphonate) (Bergh et al. 1993, Gaytan et al. 1994a,b, 1995a,b,c). Because clodronate-containing liposomes are known to induce apoptosis in macrophages in vitro and in vivo (Naito et al. 1996), and because macrophages have been shown to release substances that regulate testosterone production by Leydig cells, it is logical to speculate that these altered levels of testosterone are a result of reduced levels of macrophage-derived paracrine molecules that act on Leydig cells. While the Leydig cells exhibit normal morphological characteristics following liposome injections, little is known concerning possible direct effects of the clodronate-containing liposomes on these cells. Therefore, the purpose of the present study was to determine if liposomes containing clodronate have a direct effect on Leydig cells in vitro.

Materials and Methods

Overall design

Our first approach was to test the effects of liposomes containing clodronate and liposomes containing PBS (‘blank’ liposomes) on the viability of macrophages and Leydig cells grown in culture. We next wanted to determine if treatment with liposomes had a direct effect on testosterone production by the Leydig cells. Finally, we studied the effect of free clodronate on testosterone production.

Cell isolation

Testicular macrophages and Leydig cells were isolated as previously described from adult Sprague–Dawley rats (Hutson et al. 1996). Briefly, decapsulated testes were
digested with collagenase at 100 U/ml medium (minimal essential medium plus 0·1% BSA and 100 U/ml penicillin and 100 μg/ml streptomycin, Sigma Chemical Co., St Louis, MO, USA) in a shaking waterbath. The macrophages were isolated by preferential attachment to 35 mm culture dishes and the unattached cells containing the Leydig cells were rinsed from the dishes and centrifuged through a 55% Percoll gradient. Cells from fractions at a density range of approximately 1·09–1·12 were then washed from the Percoll and plated separately into 35 mm dishes in 1·5 ml medium. Densities were estimated using separate gradients containing colored density marker beads (Sigma) without interstitial cells (Hutson 1988). The cells were maintained at 34 °C in an atmosphere of 95% air and 5% CO₂. The macrophages were approximately 90–95% pure, while the Leydig cells were enriched to approximately 70–80% (Hutson 1988).

**Liposomes**

Liposomes were prepared by the method of Van Rooijen & Sanders (1994). This procedure produces large multilamellar liposomes which are more efficient in inducing apoptosis of macrophages than smaller unilamellar liposomes. Briefly, the liposomes were prepared by dissolving 86 mg phosphatidylcholine and 8 mg cholesterol in 10 ml chloroform (1:1) in a round bottom flask. The organic phase was evaporated. The remaining film was then dispersed by adding either 10 ml PBS (blank) or clodronate (0·6 mM). After 2 h, this solution was sonicated for 3 min and then incubated an additional 2 h at room temperature. The liposomes were washed in PBS and stored under nitrogen until use (within 2 weeks). Liposomes prepared in this manner contain approximately 1% of the clodronate solution within which they are prepared yielding a final concentration of 5 mg/ml. Because this concentration represents an estimate, doses of clodronate-containing liposomes are described as volumes rather than as concentrations.

**Assay for cell viability**

Testicular macrophages were maintained in culture for 1 h and then liposomes containing either PBS (blank) or clodronate were added in the following doses: 0, 5, 20, 50, and 100 μl. The volume of medium in all dishes was adjusted to 1·1 ml. The cells were maintained in culture for an additional 18 h, and then MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) was added at 0·5 mg/ml. Living cells with active mitochondrial dehydrogenases become blue when incubated with MTT for 3 h (Carmichael et al. 1987). The number of viable cells (blue cells) per dish was then estimated using an inverted phase contrast microscope fitted with a gridded eyepiece calibrated to a stage micrometer. Twenty grids/dish were counted using a × 20 objective.

After determining the range of doses of liposomes that would kill the macrophages as described above, we then widened this range for testing on Leydig cells so that we could most accurately determine if there was a preferential effect on macrophages. For these studies, the Leydig cells were isolated as described above and maintained in culture overnight. Either blank or clodronate-containing liposomes were then added in the following doses: 0, 50, 100, 200 or 500 μl. The final volume of medium was adjusted to 1·5 ml. Viability was determined as described above for the macrophages.

**Determination of Leydig cell function**

Leydig cells were cultured overnight as described above and treated with clodronate-containing liposomes (0, 100, 200, or 500 μl) for 18 h. The medium was then removed and centrifuged at 10 000 g to remove the liposomes. The supernatant was then assayed for testosterone using a commercial RIA obtained from Diagnostics Products (Los Angeles, CA, USA) as previously described (Hutson et al. 1996). Because the liposomes are lipophilic and represent a large percentage of the medium, we were concerned that they would partition testosterone thereby yielding low estimates of the testosterone concentration. To control for this possibility, we treated various dilutions (12·5–250 ng/ml) of testosterone in 25 μl volumes with 100 μl of liposomes in the absence of cells to determine if accurate measurements could be made in the presence of liposomes. Following incubation for 3 h at 22 °C with periodic mixing, the samples were centrifuged (10 000 g for 10 min), the pellet was extracted with 1 ml ether, and both the ether extract and the supernatant were assayed for testosterone.

As is shown below, we found that the liposomes adsorbed testosterone from the medium causing falsely low measurements. Therefore we also tested the direct effects of liposomes on testosterone production by Leydig cells at various times following removal of the liposomes. Liposomes were removed by washing three times with 1 ml culture medium and the cells incubated an additional 6, 24, and 48 h post-liposome treatment. Culture medium was then removed and assayed for testosterone. At 72 h post-liposome treatment, the cultures were stained for 3β-hydroxy steroid dehydrogenase Δ5-Δ4 isomerase (3β-HSD) activity (Hutson 1988).

Finally, we tested the effects of free clodronate on testosterone production. For these studies, clodronate was prepared in culture medium at the same concentration as would be represented by an equal volume of the lipidosome preparation. The amount of testosterone produced by Leydig cells was then determined following 24 and 48 h of
Viability was also determined following the 48 h time point as described above.

**Statistical methods**

All experiments were done with 3 or 4 replicates per treatment group. Results shown in graphs represent treatment group means ± s.e.m. Differences in cell viability between liposome-treated and control cultures were determined to be significant \((P \leq 0.05)\) by Chi-square analysis of the proportions of live (stained) and dead (unstained) cells. Differences in testosterone concentration between liposome-treated and control cultures were determined to be significant \((P \leq 0.05)\) by Fisher’s LSD multiple comparison test following analysis of variance. All descriptive and comparative statistics were computed using Statview SE software (Abacus Concepts, Inc., Berkeley, CA, USA).

**Results**

The first studies were designed to determine the effect of liposomes on viability of macrophages and Leydig cells. We found that clodronate-containing liposomes killed testicular macrophages in a dose–dependent manner following 18 h exposure while blank liposomes had no effect at any dose tested (Fig. 1). In contrast, Leydig cell viability was not significantly altered by clodronate-containing liposomes at a dose 40 times that found to kill testicular macrophages (Fig. 2). However, at the highest dose (500 µl), which resulted in a very dense layer of liposomes covering the cells on the bottom of the dish, a significant number of Leydig cells were killed. However, this effect was not due to the clodronate, since blank liposomes also killed the Leydig cells at this dose.

We next determined the effect of liposomes on testosterone production. It was found that the concentration of testosterone in the Leydig cell cultures containing clodronate-containing liposomes for 18 h was significantly lower than controls containing no liposomes (Fig. 3). Because of the possibility that the testosterone was partitioning into the lipid portion of the liposomes, and thereby unavailable to the antibody in the RIA, we next determined if liposomes could directly adsorb exogenously added testosterone in the absence of cells. It was found that a significant portion of the testosterone that had been added to culture medium did partition into the liposomes (Fig. 4).

Since we found that liposomes interfere with the accuracy of testosterone measurement, we conducted additional studies to determine the effect of liposomes on testosterone production by Leydig cells at various times following removal of the liposomes. Under these conditions, it was found that clodronate-containing liposomes had no effect on testosterone production over the following 72 h at the doses of liposomes shown in Fig. 2 to be nonlethal to Leydig cells (Fig. 5). Only at the 500 µl dose was the amount of testosterone produced less than controls. However, when these values were expressed per functional Leydig cell (as determined by staining for 3β-HSD...
activity), there was no significant difference in testosterone production between the control and liposome-treated cultures at the 72 h period (data not shown).

Finally, we determined the effects of free clodronate on testosterone production (Fig. 6). Free clodronate had no effect on either testosterone production or the viability of Leydig cells, even at doses representing the amount of clodronate present in 500µl of liposomes, when tested for 24 or 48 h.

Discussion

Several macrophage-depletion models have been used to study the role of macrophages in the testis. In the earliest study, Huhtaniemi et al. (1986) found that testicular levels of testosterone were reduced 12 h following a single injection of silica, known to be toxic when phagocytosed. They also found that Leydig cells responded poorly to human chorionic gonadotropin (hCG) when isolated from animals that had received intratesticular injections of silica. Using electron microscopy, they demonstrated that only macrophages had phagocytosed silica indicating a selective effect on the macrophages. However, they reached the scientifically sound conclusion that while it is possible that these observations were due to removal of macrophages, they may also have been due in part to direct effects on Leydig cells. In 1993, Bergh et al. conducted similar studies using an elegant new system to deplete the testis of macrophages. This involved intratesticular injection of liposomes containing clodronate.
While neither clodronate nor liposomes are thought to be toxic when used singly, they are very effective in killing macrophages by apoptosis when the clodronate is contained within the liposomes (Van Rooijen 1989, Naito et al. 1996). Although the exact mechanism of action of clodronate is unknown, it appears to be at least in part mediated by its ability to bind calcium and the presence of chlorine groups (Van Rooijen 1989). Bergh et al. (1993) found that testosterone secretion declined in the testis of unilaterally injected rats while testosterone secretion increased in the contralateral saline-injected control testis. Those two studies were the first in vivo studies to confirm the earlier in vitro studies concerning the stimulatory effects of macrophages on Leydig cells (Yee & Hutson 1985). Gaytan and colleagues have recently published a series of studies using this model system and have made the following highly significant observations: (1) macrophages are involved in Leydig cell development (Gaytan et al. 1994a, b, c), (2) Leydig cells respond poorly to hCG following removal of macrophages (Gaytan et al. 1995a, b), (3) macrophages prevent the inflammation that follows removal of Leydig cells by ethylene dimethane sulfonate (Gaytan et al. 1995c), and (4) factors from macrophages have an effect at the pituitary by inhibiting LH secretion, and at the level of the testis to stimulate steroidogenesis (Gaytan et al. 1996). In those studies it was found that saline, or saline containing liposomes had little or no effect on Leydig cells. However, those studies had not eliminated the possibility that the clodronate-containing liposomes may also have a direct effect on Leydig cells. It was for this reason that the present studies were conducted.

In the first phase of our studies we established that there is a wide margin of safety concerning the dose of clodronate-containing liposomes needed to kill Leydig cells versus macrophages. For example, while a dose of 5 µl clodronate-containing liposomes had an effect on macrophage viability, it required 500 µl to kill Leydig cells. This dose (500 µl) of liposomes completely covered the cells. It is speculated that this created a physical barrier that prevented the cells from receiving proper nutrition from the medium. In further support of this speculation was the finding that blank liposomes had a similar effect at this extremely high dose. Since clodronate-containing liposomes had no effect on Leydig cell survival, it seems that the doses used in their in vivo studies were in the same range as the lower doses (up to 200 µl) used in our in vitro studies.

In the second phase of our studies, we demonstrated that liposomes are capable of adsorbing or partitioning testosterone away from the aqueous phase, even in the absence of cells. Fresh medium was added to the cells and testosterone measured after an additional 24 h of culture (48 h time point). The percentage of viable cells was also determined using MTT after the 48 h time point. (A) Viability following treatment with clodronate for 48 h. (B) Testosterone production following treatment with clodronate.

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In the second phase of our studies, we demonstrated that liposomes are capable of adsorbing or partitioning testosterone away from the aqueous phase, even in the absence of cells. If this phenomenon also occurs in vivo, it may result in lowered intratesticular levels of testosterone, at least during the first few hours after injection.

While the studies of Gaytan and colleagues demonstrated that blank liposomes had no effect on Leydig cell survival, it was difficult to determine if they also had a direct effect on testosterone production by the Leydig cells. In the last phase of our studies we found that liposomes have no direct effect on testosterone production by Leydig cells even at doses 40 times higher than those found to kill testicular macrophages. While a reduction in
testosterone production (expressed on a per dish basis) was observed at the 500 µl dose (Fig. 5), it was not apparent when expressed per Leydig cell, indicating that the reduction was due to the lethal nonspecific effect of liposomes at this high dose. It is considered a nonspecific effect since blank liposomes also killed Leydig cells at this dose (Fig. 2). Furthermore, macrophages in vivo remove the liposomes within a few hours while the liposomes remained in direct contact with the Leydig cells for approximately 18–20 h in the in vitro conditions of the present study.

In the final phase of our studies, we found that ‘free’ clodronate had no effect on testosterone production. Therefore, it is unlikely that the clodronate released from dying macrophages in vivo would have an effect on adjacent Leydig cells. It seems reasonable that Leydig cells would be at least equally resistant to clodronate or clodronate-containing liposomes in vivo, an environment more suitable to Leydig cell maintenance than in vitro conditions used in these studies.

In summary, these data provide additional strong evidence that the in vivo model employing liposomes to deplete macrophages from the testis is a very useful and valid approach to understanding the role of macrophages in the testis.

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