The *In Vitro* Effect of Calcitriol on Parathyroid Cell Proliferation and Apoptosis

ANTONIO CANALEJO,* YOLANDA ALMADÉN,* VICENTE TORREGROSA,†
JOSE C. GOMEZ-VILLAMANDOS,‡ BLANCA RAMOS,.§ JOSE M. CAMPISTOL,†
ARNOLD J. FESENENFELD,¶ and MARIANO RODRÍGUEZ*  

*Research Unit and Nephrology Service, Reina Sofia University Hospital, Córdoba; †Nephrology Service, Hospital Clinic, Barcelona; ‡Department of Pathology, Veterinary Faculty, University of Córdoba, Córdoba; and §Nephrology Service, Carlos Haya Hospital, Malaga, Spain; and †Department of Medicine, West Los Angeles VA Medical Center and UCLA, Los Angeles, California.

**Abstract.** Calcitriol treatment is used to reduce parathyroid hormone levels in azotemic patients with secondary hyperparathyroidism (HPT). Whether long-term calcitriol administration reduces parathyroid gland size in patients with severe secondary hyperparathyroidism is not clear. The aim of the study was to evaluate *in vitro* the effect of calcitriol on parathyroid cell proliferation and apoptosis in normal parathyroid glands and in adenomatous and hyperplastic human parathyroid glands. 

Freshly harvested parathyroid glands from normal dogs and hyperplastic and adenomatous glands from patients with secondary (2°) and primary (1°) HPT undergoing parathyroidectomy were studied. Flow cytometry was used to quantify the cell cycle and apoptosis of parathyroid cells. Apoptosis was also evaluated by DNA electrophoresis and light and electron microscopy. In normal dog parathyroid glands, culture with calcitriol (10^{-10} to 10^{-7} M) for 24 h produced a dose-dependent inhibitory effect on the progression of cells into the cell cycle and into apoptosis. When glands from patients with 2°HPT were cultured for 24 h, only high calcitriol concentrations (10^{-7} M) inhibited the progression through the cell cycle and the induction of apoptosis. In parathyroid adenomas (1°HPT), even a high concentration of calcitriol (10^{-7} M) had no significant effect on the cell cycle or apoptosis. The present study shows that *in vitro*, calcitriol inhibits in a dose-dependent manner in normal parathyroid glands both parathyroid cell proliferation and apoptosis. However, in secondary hyperplasia, only high concentrations of calcitriol inhibited cell proliferation and apoptosis. In 1°HPT, even high concentrations of calcitriol had no effect. Because calcitriol simultaneously inhibits both cell proliferation and apoptosis, a reduction in the parathyroid gland mass may not occur as a direct effect of calcitriol treatment.

Most dialysis patients develop secondary hyperparathyroidism (2°HPT), which is characterized by high parathyroid hormone (PTH) levels and hyperplastic parathyroid glands. The mechanisms that drive parathyroid cells to proliferate are not clear, but it is generally accepted that a deficiency of calcitriol contributes to the development of parathyroid hyperplasia (1,2). In parathyroid cells, calcitriol acts through the vitamin D receptor (VDR) (3) to inhibit PTH mRNA expression (4,5). Calcitriol has also been shown to inhibit parathyroid cell proliferation (6) by decreasing the expression of the proto-oncogene c-myc (7). In both uremic animals (6) and patients with early renal failure (8), the administration of calcitriol has been shown to prevent the development of 2°HPT. In many patients with fully developed 2°HPT, calcitriol administration reduces the PTH level (9), but patients with severe 2°HPT often fail to respond to calcitriol (10,11). Such a failure may be due to several factors, including a decrease in the number of VDR (12–15) and the presence of hyperphosphatemia (16). In addition, severe 2°HPT is often characterized by the presence of nodular hyperplasia, which may be associated with autonomous monoclonal growth (17).

It is generally accepted that vitamin D compounds are useful in controlling 2°HPT. However, whether calcitriol induces regression of parathyroid gland hyperplasia is controversial (18–20). Apoptosis is the usual mechanism by which cells are deleted from hyperplastic tissues (21). To reduce parathyroid gland size, it would be necessary both to decrease cell proliferation and to increase cell death by apoptosis so that the net result is a decrease in the number of parathyroid cells. The aim of the present study was to evaluate the *in vitro* effect of calcitriol on parathyroid cell proliferation and apoptosis in freshly harvested normal, adenomatous, and hyperplastic parathyroid glands.

**Materials and Methods**

The study was performed using freshly excised parathyroid glands from normal dogs and from humans who required parathyroidectomy as a result of primary hyperparathyroidism (1°HPT) and azotemia-induced 2°HPT (12 glands from 12 patients with 1°HPT caused by adenomas and 30 glands from 16 patients with 2°HPT). Immediately
after resection, a piece of parathyroid tissue from each gland was maintained in culture medium at 4°C until the experiments were performed. In patients with 1°HPT, the mean PTH and serum calcium concentrations were 126 ± 13 pg/ml and 11.7 ± 0.3 mg/dl; in patients with 2°HPT, the mean PTH and serum calcium were 865 ± 122 pg/ml and 11.6 ± 0.2 mg/dl. The latter patients had severe 2°HPT that failed to respond to calcitriol therapy. Because normal human parathyroid glands were not readily available for study, normal dog parathyroid glands were obtained from euthanized animals (2 to 8 yr of age) from the City Animal Control Service. These dogs were individually housed during a period of 20 d awaiting adoption. During this time, the dogs were fed a 1.2% calcium and 0.8% phosphorus diet containing 1600 IU/kg vitamin D. At euthanization, the two superior parathyroid glands were excised and maintained in culture medium at 4°C until the experiments were performed. The parathyroidectomy required less than 5 min.

Incubation Conditions

Experiments were performed using parathyroid tissue slices instead of dispersed parathyroid cells so that tissue architecture was maintained. Parathyroid glands were cut into small pieces of approximately 1 mm² and placed in individual wells (24 well dishes from Nunclon Delta SL, InterMed, Roskilde, Denmark) with constant shaking at 37°C and placed in an incubator with a humid atmosphere. The incubation medium was buffered (pH 7.4) and contained 125 mM NaCl, 5.9 mM KCl, 0.5 mM MgCl₂, 1 mM Na₂HPO₄, and Na₂H₂PO₄ (1:2 ratio), 1 mM Na-pyruvate, 4 mM glutamine, 12 mM glucose, and 25 mM HEPES. Insulin 0.1 IU/ml, bovine serum albumin 0.1%, penicillin G 100 IU/ml, and streptomycin 100 μg/ml were added to the medium. CaCl₂ was added to achieve a final target ionized calcium concentration of 1.25 mM as measured with a selective electrode (634 Ca/pH analyzer, Ciba Corning, Essex, UK). Ionized calcium concentration was also measured in each well after the incubation module to discriminate cell aggregates. This method measured near the diploid peak. Calciotriol was added to the medium to achieve concentrations ranging from 10⁻¹⁰ to 10⁻⁷ M. To quantify the amount of free calcitriol, we filtered the medium through a 5000-MW cutoff and calciotriol was measured by RIA (Gamma 1,25-Dihydroxyvitamin D, IDS, Boldon, UK). As compared with total calcitriol, the concentration of free calcitriol was decreased by one order of magnitude.

Evaluation of Apoptosis

Apoptosis of parathyroid cells was evaluated by DNA electrophoresis, electron and light microscopy, and flow cytometry.

DNA Electrophoresis. DNA fragmentation, a characteristic feature of cell apoptosis, was documented by the electrophoretic separation of total DNA using a previously described method (22). Briefly, DNA was isolated after consecutive treatments with proteinase K and RNAase in a buffer containing 50 mM Tris-HCl, 10 mM ethylenediaminetetraacetate, and 0.05% sodium lauril sarcosinate. The electrophoresis of DNA was performed at a constant voltage of 40 V in horizontal 2% agarose gels. The bands of DNA were visualized with ethidium bromide staining.

Light and Electron Microscopy. Light microscopy was used to identify apoptosis and also to discriminate between nodular and diffuse hyperplasia. The pieces of parathyroid tissue were fixed in 10% phosphate-buffered saline (PBS)-buffered formaldehyde and embedded in paraffin. Tissue blocks were cut at 5 μ and stained with hematoxylin and eosin. Apoptosis was also demonstrated by electron microscopy, for which the tissues were fixed with 2.5% glutaralde-
Results

Normal Dog Parathyroid Tissue

Because normal human parathyroid tissue was not readily available, normal parathyroid tissue from normal dogs was studied.

Cell Cycle and Apoptosis in Normal Parathyroid Cells in Culture. In freshly harvested parathyroid tissue from dogs, there were relatively few cells in the S phase of the cell cycle (0.4 ± 0.1%) and few cells undergoing apoptosis (0.2 ± 0.1%) (Table 1). After a 24-h incubation in a culture medium without calcitriol, the number of cells progressing to the S phase increased to 8.4 ± 1.3% (P < 0.01) and cells undergoing apoptosis increased to 12.4 ± 1.8% (P < 0.01). The analysis by flow cytometry, used to quantify apoptosis, showed the hypodiploid peak (sub G0/G1 peak), which corresponds to cells with DNA fragmentation (Figure 2C) showed cellular and nuclear shrinkage with intact cell membrane; nuclear changes included pyknosis, and fragmentation with formation of apoptotic bodies (Figure 2B). By light microscopy, apoptotic cells were mixed with normal neighboring cells. The electron microscopy (Figure 2C) showed cellular and nuclear shrinkage with intact cell membrane; nuclear changes included chromatin condensation and fragmentation. Occasional apoptotic bodies surrounded by a cellular membrane were also present.

Cell apoptosis is an active process that requires protein synthesis. In a separate set of experiments, the addition of 10 μM cycloheximide, an inhibitor of protein synthesis, resulted in a decrease in apoptotic cells from 12.1 ± 2.7 to 4.7 ± 1.3% (P < 0.01).

Effect of Calcitriol on Cell Cycle and Apoptosis in Normal Parathyroid Tissue. The effect of the addition of calcitriol (from 10^{-11} to 10^{-7} M) to the culture medium for 24 h is shown in Figure 3, A and B. Even a low calcitriol concentration (10^{-11} and 10^{-10} M) reduced the progression to the S phase and to apoptosis. As the calcitriol concentration was increased from 10^{-9} to 10^{-8} M, a further suppression of cells entering the cell cycle and apoptosis was observed. Moreover, a significant correlation (r = 0.95, P < 0.01) was observed at the different calcitriol concentrations between the number of cells in S phase and those undergoing apoptosis.

Hyperplastic Human Parathyroid Tissue

Freshly harvested parathyroid glands from patients with 1°HPT and 2°HPT undergoing parathyroidectomy were studied.

Cell Cycle and Apoptosis in Adenomatous and Hyperplastic Human Parathyroid Tissue in Culture. The data on the cell cycle and apoptosis in the freshly obtained adenomatous and hyperplastic human tissue is summarized in Table 2. In parathyroid adenomas (1°HPT), the proliferative activity was variable but still was significantly greater than in 2°HPT; the percentage of cells in the G0/G1 phase was significantly decreased in 1°HPT as compared with 2°HPT (81.5 ± 5.7% versus 92.0 ± 1.0%, P < 0.05), and the percentage of cells in S phase also tended to be greater in 1°HPT than in 2°HPT (3.0 ± 1.4% versus 1.1 ± 0.3%, P = 0.06). In 2°HPT, the percentage of cells in the G0/G1 phase tended to be less in nodular than in diffuse hyperplasia (90.2 ± 1.5% versus 93.6 ± 1.3%, P = 0.08). In both nodular and diffuse hyperplasia, the percentage of cells in apoptosis was low and there was no difference between the two types of hyperplasia.

In adenomatous and hyperplastic human parathyroid tissue, the results were similar to those in normal parathyroid glands from dogs. A 24-h culture without calcitriol increased entry into the cell cycle. The S phase increased to 4.5 ± 1.8% (P < 0.05) in 1°HPT and to 6.9 ± 1.1% (P < 0.01) in diffuse hyperplasia and to 7.4 ± 1.4% (P < 0.01) in nodular hyperplasia. The entry into the cell cycle was accompanied by a concomitant increase in cells undergoing apoptosis to 9.3 ± 1.4% (P < 0.01) in 1°HPT and in 2°HPT to 12.8 ± 1.7% (P < 0.01) in diffuse hyperplasia and to 11.9 ± 2.1% (P < 0.01) in nodular hyperplasia. A significant correlation was also observed between the percentage of cells in the S phase and those undergoing apoptosis (r = 0.75, P < 0.01).

Effect of Calcitriol on Cell Cycle and Apoptosis in Human Hyperplastic Parathyroid Tissue. Studies were performed in parathyroid glands from patients with 1°HPT and 2°HPT to evaluate the effect of a 24-h culture with calcitriol on the cell cycle and apoptosis. The in vitro response of hyperplastic glands from patients with 2°HPT to calcitriol concentrations ranging from 10^{-10} to 10^{-7} M is shown in Figure 3, C and D. In contrast to the results in normal dog parathyroid glands, only the highest calcitriol concentration (10^{-7} M) was able to reduce the percentage of cells in the S phase (6.2 ± 0.7% to 3.8 ± 0.6%, P < 0.01) and this was accompanied by a decrease in the percentage of apoptotic cells (11.4 ± 1.3% to 7.6 ± 1.4%, P < 0.01).

When the in vitro response to calcitriol in parathyroid glands

Table 1. Percentage of cells in different phases of the cell cycle and apoptosis in normal dog parathyroid cells

<table>
<thead>
<tr>
<th></th>
<th>% G0/G1 Phase</th>
<th>% S Phase</th>
<th>% G2 + M Phase</th>
<th>% Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before culture</td>
<td>95.9 ± 0.9</td>
<td>0.4 ± 0.1</td>
<td>3.7 ± 0.4</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>24-h culture</td>
<td>87.4 ± 1.9(^a)</td>
<td>8.4 ± 1.3(^a)</td>
<td>4.2 ± 0.6</td>
<td>12.4 ± 1.8(^a)</td>
</tr>
</tbody>
</table>

\(^a\) P < 0.01 versus before culture; n = 14.
with nodular versus diffuse hyperplasia was compared, the response to calcitriol was similar in both types of secondary hyperplasia (Figure 4). Only at high calcitriol concentrations ($10^{-7}$ M) was a decrease in cell entry into the S phase and a decrease in cells undergoing apoptosis observed. In parathyroid adenomas (1°HPT), calcitriol concentrations of $10^{-9}$ and $10^{-7}$ M had no significant effect on the proportion of cells in the S phase of the cell cycle ($4.7 \pm 1.8\%$ cells in control versus

**Figure 1.** Analysis of parathyroid cell DNA distribution by flow cytometry. The experiments were performed using normal dog parathyroid tissue. (A) Freshly harvested parathyroid cells isolated before tissue incubation. (B) After a 24-h tissue incubation, the DNA histogram shows the hypodiploid peak (arrow), which is formed by cells undergoing apoptosis.

**Figure 2.** Documentation of parathyroid cell apoptosis after 24 h of parathyroid tissue incubation in the absence of calcitriol. The experiments were performed using normal dog parathyroid tissue. (A) Fragmentation of DNA at 24 h as compared with intact DNA at time 0 (before the culture). (B) Light microscopy of parathyroid tissue after a 24-h incubation. The arrows represent single apoptotic parathyroid cells with nuclear pyknosis and fragmentation. (C) Electron microscopy of an apoptotic parathyroid cell showing cytoplasmic shrinkage, condensed nuclear chromatin, and intact cell membrane. Magnification, 12,000×.
5.2 ± 2.2% and 4.6 ± 1.9% in calcitriol 10^{-9} and 10^{-7} M, respectively) and apoptosis (9.1 ± 1.2% in the control versus 6.9 ± 2.0% and 7.0 ± 1.8% with calcitriol 10^{-9} and 10^{-7} M, respectively).

**Discussion**

The present study evaluates the *in vitro* effect of calcitriol on the cell cycle and apoptosis of cells from normal dog parathyroid glands and from human parathyroid glands with secondary hyperplasia and with primary adenoma. The results of the study show that in normal parathyroid tissue, calcitriol decreases both parathyroid cell proliferation and apoptosis. In parathyroid tissue from humans with 2°HPT, an inhibition of proliferation was observed only at high concentrations of calcitriol. The inhibition of cell proliferation induced by a high calcitriol concentration was also associated with a decrease in cell apoptosis. Cells from parathyroid adenomas (1°HPT) showed no response to calcitriol.

In freshly harvested parathyroid tissue from normal dogs, the percentage of parathyroid cells in the S phase of the cell cycle and those undergoing apoptosis was low. These results are in agreement with previously reported data from normal parathyroid glands (24–27). By contrast, after 24 h of tissue culture without calcitriol, a significant number of cells progressed into the S phase and this was associated with an increase in apoptosis. Furthermore, a significant correlation was observed between the percentage of cells in the S phase and the percentage of apoptotic cells. From this significant

**Table 2.** Cell cycle and apoptosis values (% cells) in parathyroid glands from patients with 1°HPT and 2°HPT

<table>
<thead>
<tr>
<th></th>
<th>% G0/G1 Phase</th>
<th>% S Phase</th>
<th>% G2 + M Phase</th>
<th>% Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1°HPT (n = 12)</td>
<td>81.5 ± 5.7</td>
<td>3.0 ± 1.4</td>
<td>15.5 ± 7.1</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>2°HPT (n = 30)</td>
<td>92.0 ± 1.0b</td>
<td>1.1 ± 0.3</td>
<td>6.9 ± 0.6</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>2°HPT-diffuse (n = 16)</td>
<td>93.6 ± 1.3b</td>
<td>0.8 ± 0.4b</td>
<td>5.6 ± 1.2</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>2°HPT-nodular (n = 14)</td>
<td>90.2 ± 1.5b</td>
<td>1.4 ± 0.5</td>
<td>8.4 ± 1.5</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

*1°HPT, primary hyperparathyroidism; 2°HPT, secondary hyperparathyroidism; 2°HPT-diffuse, diffuse hyperplasia; 2°HPT-nodular, nodular hyperplasia.

*P < 0.05 versus 1°HPT.
correlation, it may be assumed that cells that enter but do not complete the cell cycle eventually undergo apoptosis. Parathyroid cells, as do many other cell types, tend to proliferate in vitro (24), and it has been proposed that the same stimuli that induce cell activation and proliferation may also lead to apoptosis (28). This phenomenon may be particularly relevant in tissues with low cell turnover such as the parathyroids. It is interesting that cells, such as neurons, that have irreversibly exited the cell cycle will undergo apoptosis in response to the same molecular events that in dividing cell populations lead to proliferation. Thus, the relatively high rate of proliferation observed in culture made this in vitro model suitable for determining an effect of calcitriol on parathyroid cell proliferation and apoptosis.

Our results show that in normal parathyroid tissue, the addition of calcitriol to the tissue culture produced an inhibition of cell proliferation and of apoptosis. This effect of calcitriol was dose dependent and was observed even at a low calcitriol concentration (10^{-10} M). Previous reports have shown that calcitriol reduces parathyroid cell proliferation (6) by decreasing proto-oncogene c-myc expression (7). This gene is involved in the regulation of the cell cycle (by modulating the progression of cells from the G1 into the S phase) and may also promote apoptosis in activated cells when it is anomalously expressed (28). Thus, the deficiency of calcitriol in parathyroid tissue cultures may cause both the progression into the cell cycle and the induction of apoptosis via a disinhibition of c-myc expression. The addition of calcitriol to the culture would have the opposite effect. Thus, it is not surprising to observe that the decrease in parathyroid cell proliferation induced by calcitriol is accompanied by a reduction in apoptosis.

The analysis of the freshly obtained parathyroid tissue from parathyroidectomized patients showed that adenomas had a higher proliferative activity than did secondary hyperplasia. Between the two forms of secondary hyperplasia, nodular hyperplasia had slightly higher proliferative rates than did diffuse hyperplasia. These findings are similar to those previously reported by others (30–32). In adenomatous and hyperplastic human parathyroid tissue, the number of apoptotic cells was low and was not significantly different between 1°HPT and 2°HPT. We cannot be certain that the percentage of apoptotic cells in hyperplastic parathyroid tissue was increased as compared with normal parathyroid tissue because normal parathyroid glands were not available for study. However, a previous publication reported that the percentage of apoptotic cells was greater in glands from patients with 2°HPT than in normal parathyroid tissue (25). In the literature, non-uniform data have been reported about the rates of apoptosis in 1°HPT and 2°HPT. Whereas some authors reported values as high as 5.3% and 9.5% of apoptotic cells for 1°HPT and 2°HPT, respectively (25), others have observed 0.35% and 0.1%, respectively (33); the latter values are close to our results. These differences may be due to the different methods used to quantify apoptosis.

The in vitro response of parathyroid tissues from secondary...
Calcitriol on the cell cycle and apoptosis in nodular or diffuse hyperplasia (32). More autonomous in function and growth than parathyroid glands, they have been reported to have a greater decrease in VDR and to be more likely to respond to calcitriol treatment and the proposed mechanism was a decrease in the number of cells as a result of an increase in cell apoptosis (20,34). However, other authors have not been able to demonstrate a decrease in parathyroid gland size in patients with 2°HPT treated with calcitriol (10,18,19). In fact, the effect of calcitriol on the cell cycle and apoptosis that we have observed in the in vitro setting is similar to that in vivo, then the administration of calcitriol should not directly reduce the size of the parathyroid glands. The decrease in parathyroid cell proliferation induced by calcitriol might stop parathyroid gland growth. However, to reduce the mass of parathyroid tissue, it would be necessary to increase the rate of apoptosis; the results of our in vitro experiments suggest that calcitriol at concentrations similar to those observed in vivo failed to induce apoptosis of parathyroid cells. Because of the coupling between proliferation and apoptosis, it seems unlikely, on the basis of our in vitro findings, that calcitriol changes the balance between the two processes. Moreover, it is commonly observed that in a number of renal transplant patients in whom calcitriol levels return to normal, parathyroid hyperplasia does not regress and eventually parathyroidectomy is required. This clinical observation suggests that in patients with advanced 2°HPT, the normalization of serum calcitriol levels after transplantation is not followed by a significant increase in parathyroid cell apoptosis. However, it must be stressed that the parathyroid glands used in this study were obtained from patients with severe 2°HPT who did not respond to calcitriol in vivo. Thus, regardless of the histologic nature of the hyperplasia, it is also possible that parathyroid glands from patients with earlier stages of 2°HPT, who respond to calcitriol in vivo, would be more sensitive to calcitriol in vitro.

Because parathyroid glands with nodular hyperplasias have been reported to have a greater decrease in VDR and to be more autonomous in function and growth than parathyroid glands with diffuse hyperplasia (15), we compared the effect of calcitriol on the cell cycle and apoptosis in nodular versus diffuse hyperplasia. Our results showed that there were no differences between the in vitro response in both types of hyperplasia. In earlier stages of parathyroid hyperplasia, cells from diffuse hyperplasia might be more susceptible to modulation by calcitriol (6). However, the present study was performed in glands from parathyroidectomized patients with advanced 2°HPT. Other authors (17) have detected monoclonal growth in glands with diffuse hyperplasia from parathyroidectomized patients with advanced 2°HPT, and it is conceivable that virtually all glands from patients with uncontrolled 2°HPT could be considered as nodular hyperplasias. A recent report showed a high proliferative capacity in parathyroid cells autotransplanted to the forearm whether the graft included areas of nodular or diffuse hyperplasia (32).

In conclusion, we have shown in an in vitro model that calcitriol inhibited both proliferation and apoptosis of parathyroid cells from normal dogs. However, in contrast to the normal dog, the response to calcitriol in human parathyroid glands from patients with 1°HPT and those with advanced 2°HPT was marginal. The failure to respond in vitro is similar to the behavior of these parathyroid glands in vivo. Because of coupling between proliferation and apoptosis, it seems unlikely, on the basis of our in vitro findings, that calcitriol affects the balance between the two processes. Thus, a reduction of the parathyroid gland mass should not be expected as a direct effect of calcitriol treatment in patients with severe 2°HPT.

Acknowledgments

The work presented here was supported by Dirección General de Ciencia y Tecnología (Grant PM 96-0184), Fondo Investigación Sanitaria (99-0768), Consejería de Salud de la Junta de Andalucía (Grant JA97/156), Fundación Hospital Reina Sofia-CajaSur and Baxter Extramural Grant. A. Canalejo is supported by a Becas de Perfeccionamiento from Instituto de Salud Carlos III (Exp.: 97/4336).

References


Access to UpToDate on-line is available for additional clinical information at http://www.jasn.org/