Effect of aluminium on calcium-sensing receptor expression, proliferation, and apoptosis of parathyroid glands from rats with chronic renal failure

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Background. To assess the effect of aluminium on the calcium-sensing receptor expression, proliferation, and apoptosis in parathyroid glands from rats with chronic renal failure, 2½-month-old male Wistar rats were 7/8 nephrectomized.

Methods. Eight weeks after surgery the rats were divided into two groups, one receiving intraperitoneal AlCl₃, for 8 weeks and the other receiving intraperitoneal placebo. Serum Al, Ca, P, creatinine, and PTH were measured. Parathyroid glands were removed, formaldehyde-fixed, and paraffin-embedded. Calcium-sensing receptor and proliferation were detected by immunohistochemistry and apoptosis by TUNEL and propidium iodide uptake.

Results. At the end of the study, despite higher levels of serum P in the aluminium group (6.27 ± 0.63 vs. 5.56 ± 0.58 mg/dL; P = 0.045), serum PTH was lower (89.6 ± 57.7 vs. 183.1 ± 123.8 pg/mL; P = 0.059). No significant differences were found in the calcium-sensing receptor expression between groups (aluminium: 27.1 ± 7.6; placebo: 25.4 ± 3.5 RU). Rats receiving aluminium showed a significantly lower cell proliferation rate than the control rats (0.54 ± 0.69 vs. 4.43 ± 3.10 cells/mm²; P = 0.003). No apoptotic events were detected.

Conclusion. Aluminium was able to reduce the cell proliferation of the parathyroid glands. Due to the low apoptosis rate, however, it was not possible to find any change. Aluminium had no effect on the calcium-sensing receptor expression.

METHODS

Animals

The study was carried out in 2½-month-old male Wistar rats (N = 25) weighing 421 ± 53 g. The animals were housed at 25°C, fed with a standard diet (Panlab: A-04® 0.6% calcium and 0.6% phosphorous) and drinking water ad libitum. The rats were then divided into two groups, each having similar weights and renal function. The study was initiated after one week of adaptation. Chronic renal failure was induced surgically by 7/8 nephrectomy using the technique modified by Ormrod and Miller [10]. After 8 weeks of chronic renal failure, the rats were weighed, and urine and blood samples were taken in order to measure urea, calcium, phosphorous, alkaline phosphatase, and PTH. The aluminium group received 1 mg of intraperitoneal aluminium (8.94 mg AlCl₃·6H₂O) five days per week for eight weeks. The total amount of aluminium adminis-
tered was 38.5 mg/rat. The initial dose of aluminium was progressively increased (0.25, 0.5, 0.75, 1 mg/day) in order to decrease mortality [11]. The placebo group received the same volume of a saline solution with the same pH as that of the aluminium solution (pH = 2.3). After eight weeks of treatment, the rats were anesthetized by ether; urine and blood samples were then taken by cardiac puncture and the animals were sacrificed. The parathyroid glands were then extracted using a stereomicroscope (Olympus Mod SZ-ST, Tokyo, Japan). The size of the gland was measured and then fixed in 4% formaldehyde and embedded in paraffin. The whole process of extraction and measurement was performed in less than five minutes.

Biochemical analysis

Serum calcium, phosphorous, urea, and alkaline phosphatase were measured using a multichannel autoanalyzer (Hitachi 717®, Boehringer Mannheim, Mannheim, Germany). Serum PTH was measured using an IRMA Rat PTH® Kit (Nichols Institute, San Juan Capistrano, CA, USA). Serum aluminium was measured by Graphite Furnace Atomic Absorption Spectrometry (PerkinElmer®, Shelton, CT, USA) following the methods previously described [12].

Image analysis

All the measurements (size of the glands, calcium-sensing receptor, proliferation, and apoptosis) were performed using an optical microscope Mod. Polyvar from Reichert-Jung (Wetzlar, Germany) coupled to a digital video camera (Leica Microsystems Mod. Dc-100, Wetzlar, Germany). The digital images were then analyzed using an image analysis system (Leica Q500IW) in addition to a specific software program (Leica QWIN standard v.2.3, Leica microsystems).

Measurement of the parathyroid gland size

The size of the parathyroid glands were measured immediately after their extraction. Each gland was placed between two glass slides 0.014 mm thick (Fig. 1). Using this procedure, the glands were forced to be the same height and, therefore, the area of the gland was proportional to its volume. The image of each gland was captured with the microscope and saved for a later measurement. Two different operators measured the area of each gland by using the image analysis system.

Immunohistochemistry

The calcium-sensing receptor and cellular proliferation were detected by immunohistochemistry in 5-μm thick serial sections from paraffin-embedded parathyroid glands using specific antibodies and hematoxylin counterstaining (EnVision+® System, Dako, Carpinteria, CA, USA). The calcium-sensing receptor was detected using a rabbit polyclonal antibody against a 23-amino acid peptide contained in the extracellular domain of the calcium-sensing receptor (kindly provided by Dr. A. Brown and Dr. E. Slatopolsky, St. Louis, MO, USA) [13]. All the samples were processed at the same time in order to keep the possible variables of the immunostaining procedure constant. Immunohistochemical staining of the calcium-sensing receptor was quantitated as previously described [13]. The proliferation was detected using a monoclonal antibody against the proliferating cellular nuclear antigen (PCNA, clone PC-10 from Biogenex, San Ramon, CA, USA) [14]. The number of stained cells was divided by the total area of the gland so that the results were expressed as cells/mm². All the samples were stained at the same time and under the same conditions previously described for the calcium-sensing receptor.

Apoptosis

The detection of apoptosis was carried out by two different methods: terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) (TdT-FragEL®, Oncogene, La Jolla, CA, USA), and the propidium iodide uptake technique [15]. Both techniques were performed on 5-μm thick sections from paraffin-embedded parathyroid glands.

Statistical analysis

The statistical analysis was performed with the statistical package SPSS 8.0 for Windows (Microsoft Corporation, Redmond, WA, USA). The comparisons were performed using nonparametric tests for two independent samples (Mann-Whitney test), and for two related samples (Wilcoxon test). Significant differences were considered when $P < 0.05$.

RESULTS

The rats in the placebo group significantly increased their weight from the beginning to the end of the study.
Table 1. Weight and biochemical parameters of the rats (mean ± SD) before (basal) and 8 weeks after (final) administration of aluminium or placebo

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Basal</th>
<th>Aluminium</th>
<th>Final</th>
<th>Placebo</th>
<th>Aluminium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight g</td>
<td>513 ± 76</td>
<td>490 ± 43</td>
<td>533 ± 80*</td>
<td>479 ± 45</td>
<td></td>
</tr>
<tr>
<td>Urea mg/dL</td>
<td>82.3 ± 13.3</td>
<td>77.7 ± 14.8</td>
<td>83.0 ± 9.9</td>
<td>71.8 ± 14.2</td>
<td></td>
</tr>
<tr>
<td>Ca mg/dL</td>
<td>11.48 ± 0.29</td>
<td>11.45 ± 0.29</td>
<td>11.12 ± 0.31</td>
<td>10.98 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>P mg/dL</td>
<td>6.16 ± 0.60</td>
<td>5.98 ± 0.53</td>
<td>5.56 ± 0.58</td>
<td>6.27 ± 0.63*</td>
<td></td>
</tr>
<tr>
<td>PTH pg/mL</td>
<td>164 ± 155</td>
<td>126 ± 75</td>
<td>183 ± 124</td>
<td>90 ± 58</td>
<td></td>
</tr>
<tr>
<td>AP IU/L</td>
<td>305 ± 106</td>
<td>244 ± 82</td>
<td>230 ± 63</td>
<td>317 ± 250</td>
<td></td>
</tr>
<tr>
<td>Aluminium ng/mL</td>
<td>—</td>
<td>4.13 ± 3.69</td>
<td>526.11 ± 88.63*</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Area per treatment group mm²</td>
<td>—</td>
<td>3.58 ± 0.61</td>
<td>3.16 ± 0.52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AP is alkaline phosphatase.
*P < 0.03 compared with basal by Wilcoxon test
*P < 0.05 compared with placebo
*P = 0.066 compared with placebo
*P < 0.001 compared with placebo by Mann-Whitney test

(513 ± 76 vs. 533 ± 80 g; P < 0.03); however, no changes were observed in the aluminium group. Before the administration of aluminium or placebo (week 8), no significant differences were found in weight or in biochemical parameters between the two groups. At the end of the study (week 16), no differences were found in any of the parameters measured except for serum aluminium and phosphorous (Table 1). Serum phosphorous was significantly higher in the aluminium group (5.56 ± 0.58 vs. 6.27 ± 0.63 mg/dL; P < 0.05). In the aluminium group, a decrease of the serum PTH was found, although it was not significant (183 ± 124 vs. 90 ± 50 pg/mL; P = 0.066).

At the end of the study we analyzed 30 glands, 18 in the placebo group and 12 in the aluminium group. No significant differences in gland size between the two groups were found (placebo: 3.58 ± 0.61 vs. aluminium: 3.16 ± 0.52 mm²; P = 0.190).

No significant differences were found in the calcium-sensing receptor expression between both groups (placebo: 25.4 ± 3.5 vs. aluminium: 27.1 ± 7.6 relative units [RU]) (Fig. 2). The glands from the rats receiving placebo showed a significantly higher proliferation rate than the glands from rats receiving aluminium (4.43 ± 3.10 vs. 0.54 ± 0.69 cells/mm²; P < 0.005) (Fig. 2). No apoptotic events were detected in any of the glands studied.

DISCUSSION

Aluminium is able to inhibit parathyroid function in vivo and in vitro [16–20], even in cases of stimulated hypocalcemia [21]. This suggests a direct effect on PTH release, but not on its synthesis [22]. However, a recent study has shown that aluminium is, in fact, also capable of reducing the levels of PTH mRNA [23]. In our study we found that aluminium was able to reduce PTH levels by half (not statistically significant), despite increases in serum phosphorous.

Calcium is the main regulator of PTH, its effect mediated by the calcium-sensing receptor [24]. This protein has the ability to interact with polyvalent cations [5, 6], which may justify the hypothesis that aluminium has a PTH effect, acting on the calcium-sensing mechanism. Recent studies have concentrated on this possibility, sug-
gesting that aluminium could be a weak agonist of the calcium-sensing receptor, capable of activating the receptor at high concentrations [25].

In our study, we have not observed that aluminium has any effect on the calcium-sensing receptor expression, despite reaching a high concentration of serum aluminium. However, our results cannot be directly compared with the aforementioned study because both studies evaluated different effects of aluminium on the calcium-sensing mechanism. The former studied the ability of aluminium to activate the intracellular signaling pathways ending with the suppression of PTH release [25], whereas our study assessed the effect of aluminium on the expression of the receptor. Taking into account the results from both studies, it would seem that the activation of the receptor by aluminium is more likely the mechanism of PTH suppression, rather than the receptor expression. The activation of the receptor may only take place at high (non-physiologic) concentrations of aluminium [25].

We found that aluminium was capable of reducing the proliferation of parathyroid cells. This reduction was significant, despite higher serum phosphorous levels in the aluminium group, a fact that may have had the opposite effect of aluminium, instead stimulating the proliferation of parathyroid tissue [13]. The effect of high serum phosphorous was not able to overcome the inhibitory effect of aluminium on cell proliferation.

Several authors have observed a decrease in the calcium-sensing receptor protein expression levels in human parathyroid glands with a high proliferation rate [26, 27]. The same results have been observed in uremic rats that were fed a high-phosphorus diet [13]. In our study, the calcium-sensing receptor expression did not decrease, in spite of the higher phosphorus levels in the aluminium group. This may suggest that high serum aluminium may have counteracted the effect of phosphorous, impeding the fall of calcium-sensing receptor levels.

As in other studies [28–30], we were not able to detect apoptotic events in any of the groups, which might be explained by the extremely low rate of cell turnover in the parathyroid tissue. On the contrary, we have come across some indirect evidence of apoptosis. The lower proliferation rate did not correlate with a reduction in the size of the glands (3.58 ± 0.61 placebo vs. 3.16 ± 0.52 aluminium; P = 0.190). Because there was no variation in the size of the gland despite a significant reduction in the proliferation, it could be assumed that the reduction in the proliferation rate should be accompanied by a reduction in the apoptosis rate, although the latter is only a speculation.

In summary, although the inhibitory effect of aluminium on parathyroid function is clear, the mechanisms by which this occurs are not. In our study, aluminium does not appear to have a direct effect on the expression on the calcium-sensing receptor. In addition, we were also unable to demonstrate any effect of aluminium on apoptosis. On the contrary, we clearly demonstrated that aluminium is capable of diminishing cellular proliferation in the parathyroid glands. This effect may partly explain the long-term negative effect of aluminium load on parathyroid growth and function.

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