Lymphokine-induced Monocytic Differentiation as a Possible Mechanism for Hypercalcemia Associated with Adult T-Cell Lymphoma

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ABSTRACT

Patients with adult T-cell lymphoma frequently have hypercalcemia. Bone biopsies from these patients show increased numbers of osteoclasts. We hypothesized that substances produced by the malignant T-cell caused these phenomena by increasing the formation and/or activity of osteoclasts. To test this hypothesis, we cultured U937 cells in conditioned media from a clonal T-cell line derived from a patient with adult T-cell lymphoma and hypercalcemia. This conditioned media produced maturational changes in the U937 cells as evidenced by decreased proliferation, increased adherence, increased expression of complement receptors, and formation of multinucleated giant cells. These changes were synergistically enhanced by the addition of 1α,25-dihydroxyvitamin D3, which is known to promote monocyte differentiation. We also tested interleukin 2 and γ- and α-interferon to see if they were responsible for the maturational changes. We also tested interleukin 2 and γ- and α-interferon to see if they were responsible for the maturational changes. The role of calcitriol in this process was also examined.

INTRODUCTION

Adult T-cell lymphoma is a malignant proliferation of helper T-cells (15). This disorder is associated with type-C retrovirus (HTLV) infection (23) and is frequently complicated by hypercalcemia (3–5, 12). The patients with hypercalcemia characteristically have normal serum immunoreactive parathyroid hormone levels, multiple lytic lesions on skeletal X-rays, generalized increased activity on bone scan, and increased numbers of both osteoclasts and osteoblasts on bone biopsy (3–5, 12). These findings formed the basis for the hypothesis that the hypercalcemia of adult T-cell lymphoma is caused by one or more lymphokines secreted by the malignant T-cells and that they produce hypercalcemia by promoting the differentiation of osteoclast precursors and be involved in the pathogenesis of the hypercalcemia.

MATERIALS AND METHODS

Cells and Cell Culture Conditions. U937 cells were passaged every 2 to 3 days in medium consisting of Dulbecco's modified Eagle's minimal essential medium: Ham's F-12 medium (Grand Island Biological Co., Grand Island, NY) (1:1) supplemented with 5% FBS, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, penicillin, 100 units/ml, and streptomycin, 100 µg/ml. Cells were incubated at 37°C in humidified 5% CO2:95% air. Viability was determined by trypan blue exclusion. Morphology was assessed by phase-contrast microscopy. Wright's stain was used to evaluate multinucleated giant cells.

HUT-516 cells (a cell line isolated from a patient with adult T-cell lymphoma and hypercalcemia) were passaged every 3 days in RPMI 1640 (Grand Island Biological Co.) which contained 10% FBS and 1% T-cell growth factor (Meloy Laboratories, Springfield, VA). HTLV antigens p19 and p24 were found in the DNA of the patient's original malignant T-cells. The HUT-516 cell line was positive for HTLV-I by monoclonal analysis and for HTLV p19 by nucleic acid hybridization in studies performed by Dr. Bernie Poiesz, Upstate Medical Center, Syracuse, NY. Serum-free conditioned media were obtained by culturing the HUT-516 cells for 3 days in media consisting of RPMI-1640 with bovine serum albumin, 0.5%, insulin, 5 µg/ml, transferrin, 35 µg/ml, ethanolamine, 20 µM/ml, and selenium, 10⁻⁴ M. This cell suspension was centrifuged at 100 × g for 10 min, and the supernatant was removed, filtered through a 0.2-µm filter, and stored at −20°C. A control conditioned medium was obtained from U937 cells using the same procedure as above.

U937 cells were tested by Dr. Barton Haynes, Duke University, NC, for infection with human T-cell lymphoma virus after 5 and 21 days of culture in 20% HUT-516-conditioned media. The cells were assayed for the HTLV-associated antigens designated as p19 and p24 as described (16) and were found to be negative. 1,25-(OH)₂D₃ was kindly provided by Dr. M. Uskokovic (Hoffman-LaRoche, Nutley, NJ). The 1,25-(OH)₂D₃ was dissolved in ethanol and added to the cell cultures so that the ethanol concentration was less than 0.1%. IL-2 (2 µM, also known as T-cell growth factor) was purchased from Meloy Laboratories, Springfield, VA. Interleukin 1, 2 units/ml, and IFN-α, 2 units/ml, were purchased from Meloy. α-Tac were donated by Dr. Thomas Waldman...
RESULTS

HUT-516 TC-CM. Under the usual conditions, U937 cells grow in suspension with a doubling time of 24 h. Less than 3% of the cells adhere to the surface of the culture dish under these conditions. Conditioned media from U937 cells used as a "spent media" control or media containing 10 nm 1,25-(OH)2D3 decreased cell growth but did not promote adherence (Table 1). The addition of 20% TC-CM to the medium increased the number of adherent U937 cells 3-fold and decreased by 60% the total cell number after 3 days of culture. Cells of culture in the presence of both 20% TC-CM and 10 nm 1,25-(OH)2D3 caused a further doubling of the number of adherent U937 cells compared to 20% TC-CM alone. These effects were observed as early as 24 h of culture and persisted for at least 5 days.

These experiments were repeated using HUT-516 conditioned media that was obtained under serum-free conditions. Significant but less striking changes were seen (data not shown). The combination of 10^-8 M 1,25-(OH)2D3 plus 10% HUT-516 conditioned media (serum free) resulted in 20% of the total cells being adherent after 3 days of culture. Serum-free media alone used as a control produced no changes. We have reported previously that 1,25-(OH)2D3 increased the percentage of U937 cells expressing complement receptor for C3b (CR3) but not for iC3b (CR2) as determined by rosette formation (8). TC-CM either with or without 1,25-(OH)2D3 increased the percentage of cells expressing CR3 more than the reported effect of 1,25-(OH)2D3 alone and also increased the percentage of cells expressing CR2 from less than 6 to greater than 60% of the adherent cells.

Polykaryons did not form spontaneously in the U937 cell cultures. TC-CM alone or with 1,25-(OH)2D3 led to polykaryon formation in 5 to 7% of the adherent subpopulation as assessed by light microscopy. These cells contain tartrate-resistant acid phosphatase and are capable of resorbing bone.

Effects of m-IL-2 on U937 Cells. U937 cells were cultured in the m-IL-2 for up to 5 days. A concentration-dependent growth inhibition was evident during this time period (Chart 1). An increase in the number of adherent cells occurred despite the decrease in cell growth (Table 2) further suggesting maturation of the U937 cells. As in the HUT-516 conditioned media experiments, the addition of 10 nm 1,25-(OH)2D3 to the m-IL-2 resulted in a synergistic increase in adherent cell number. Maximum increases in adherence were present after 1 day of culture with m-IL-2 alone (data not shown), whereas adherence continued to increase until 3 days of culture in m-IL-2 plus 1,25-(OH)2D3-treated cells.

Our previous work (8) showed that the increase in phagocytosis and CR3 expression induced by 1,25-(OH)2D3 were concentration dependent and required supraphysiological levels to achieve statistically significant changes. In contrast, the synergistic enhancement of the m-IL-2 effects on growth and adherence occurred at physiological concentrations of calcitriol (Table 3).

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Table 1

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Total cells (×10^6)</th>
<th>Adherent cells</th>
<th>% Adherent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>91.2 ± 2.5a</td>
<td>2.98 ± 0.04</td>
<td>3</td>
</tr>
<tr>
<td>20% control conditioned from</td>
<td>67.4 ± 4.3a</td>
<td>1.84 ± 0.03</td>
<td>3</td>
</tr>
<tr>
<td>U937 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25-(OH)2D3</td>
<td>78.8 ± 2.3p</td>
<td>4.08 ± 0.06</td>
<td>5</td>
</tr>
<tr>
<td>20% TC-CM</td>
<td>36.7 ± 1.6p</td>
<td>9.24 ± 0.10p</td>
<td>25</td>
</tr>
<tr>
<td>20% TC-CM + 1.25-(OH)2D3</td>
<td>31.1 ± 1.0p</td>
<td>17.60 ± 1.2p</td>
<td>57</td>
</tr>
</tbody>
</table>

a Mean ± SE of 8 wells for each value. p < 0.01 versus control by analysis of variance and Fisher least significant difference tests.
U937 cells (10⁵) were cultured for 1 to 5 days in the above conditions. Total cells increased with the time of incubation. CR3 expression rose from 2% seen in nonadherent controls to virtually 100% of the adherent subpopulation. The presence of m-IL-2 and 1,25-(OH)₂D₃ increased the expression of all 3 receptors more than did m-IL-2 alone (Chart 2).

Analysis with flow cytometry showed that at least 35% of U937 cells had IL-2 receptors. Forty-five % of cells incubated in 10 nm 1,25-(OH)₂D₃ for 3 days expressed the receptors. Receptors for C3b (CR1) and IgG (Fc) determined by rosette formation are expressed by a small number of resting U937 cells, and receptors for iC3b (CR3) measured by the same techniques are undetectable (8). Therefore, we examined whether adherence was associated with maturation of complement receptor expression.

Chart 2. Effects of 5 to 15% IL-2 and/or 10 nM 1,25-(OH)₂D₃ on cell number.

Table 2
Effect of m-IL-2 and 1,25-(OH)₂D₃ (10 nm) on adherence after 3 days of culture.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Adherent cells (x10⁴)</th>
<th>% adherent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.87 ± 0.23</td>
<td>1</td>
</tr>
<tr>
<td>1,25-(OH)₂D₃ (10 nm)</td>
<td>2.34 ± 0.74</td>
<td>5</td>
</tr>
<tr>
<td>5% m-IL-2</td>
<td>4.38 ± 0.43</td>
<td>20</td>
</tr>
<tr>
<td>10% m-IL-2</td>
<td>3.82 ± 0.14</td>
<td>23</td>
</tr>
<tr>
<td>10% m-IL-2 + 1,25-(OH)₂D₃ (10 nm)</td>
<td>16.84 ± 0.64</td>
<td>62</td>
</tr>
<tr>
<td>15% m-IL-2</td>
<td>2.95 ± 0.09</td>
<td>25</td>
</tr>
</tbody>
</table>

*p Mean ± SE of 8 wells for each value.

*p P < 0.01 versus control.

cubated with α-Tac (1:5000 dilution) before culture with 10% m-IL-2 showed a 50% reduction in the number of adherent cells and an increase in total cell number compared to cells incubated with m-IL-2 but not exposed to α-Tac. The addition of 1,25-(OH)₂D₃ did not alter the effects of α-Tac on the cells. On repeat testing, the α-Tac again produced concentration-dependent inhibition of IL-2 effects but concentrations greater than 1:500 produced further blunting of the response.

Receptors for C3b (CR1) and IgG (Fc) determined by rosette formation are expressed by a small number of resting U937 cells, and receptors for iC3b (CR3) measured by the same techniques are undetectable (8). Therefore, we examined whether adherence was associated with maturation of complement receptor expression. Chart 2 depicts the percentage of U937 cells expressing these receptors in the adherent subpopulation of cells cultured with either m-IL-2 alone or with m-IL-2 plus 10 nm 1,25-(OH)₂D₃. The percentage of cells expressing CR1 and CR3 increased with the time of incubation. CR3 expression rose from 2% seen in nonadherent controls to virtually 100% of the adherent cells cultured in the presence of both m-IL-2 and 1,25-(OH)₂D₃. The presence of m-IL-2 and 1,25-(OH)₂D₃ increased the expression of all 3 receptors more than did m-IL-2 alone (Chart 2). Expression of the Fc receptor was decreased on the third day of incubation. This phenomenon is unexplained, but it has
LYMPHOKINE-INDUCED MONOCYTIC DIFFERENTIATION

Effects of 10% IL-2 with and without 10 nm 1,25-(OH)\(_2\)D\(_3\) on percentage of adherent U937 cells expressing CR\(_{1}\), CR\(_{3}\), and Fc receptors. U937 cells (10\(^6\)) were cultured for 1, 3, and 5 days. Nonadherent cells were removed, and the percentage of 200 cells forming rosettes with C3b-, iC3b-, or IgG-coated sheep erythrocytes were determined. Values are expressed as mean ± SE (bars).

![Chart 2](image)

been observed previously with adherent monocytes (20). Incubation of cells with m-IL-2 and 25-hydroxyvitamin D\(_3\) or 24R,25-dihydroxyvitamin D\(_3\) increased CR\(_{1}\), CR\(_{3}\), and Fc receptor expression to the same extent as that observed with the combination of m-IL-2 and 1,25-(OH)\(_2\)D\(_3\) except that higher concentrations of the metabolites were required (data not shown).

Addition of m-IL-2 enhanced receptor expression on the non-adherent subpopulation in addition to its effects on adherent cells. The percentage of U937 cells expressing CR\(_{1}\) and Fc receptors increased after 5 days of culture with m-IL-2 ± 10 nm with and without 1,25-(OH)\(_2\)D\(_3\) compared to control or 1,25-(OH)\(_2\)D\(_3\) alone (Table 5). Only m-IL-2 plus 1,25-(OH)\(_2\)D\(_3\) significantly altered CR\(_3\) expression in this subpopulation. Changes were present after 1 and 3 days of culture but were not as striking as the 5-day results (data not shown). Rosette formation by C3b- and iC3b-coated erythrocytes was inhibited by preincubating the U937 cells with anti-CR\(_{1}\) and anti-CR\(_{3}\) antibodies, respectively, confirming their specificity for CR\(_{1}\) and CR\(_{3}\) under the assay conditions used.

Adherent cells ingested complement-coated erythrocytes in addition to rosette formation. After 5 days of incubation, 11, 36, and 73% of the adherent cells incubated with 10% m-IL-2 ingested one or more erythrocytes coated with C3b, iC3b, and IgG, respectively. The addition of 10 nm 1,25-(OH)\(_2\)D\(_3\) to m-IL-2-treated cells did not increase the amount of erythrophagocytosis seen with 10% IL-2 alone. Similar changes of a lower magnitude were observed after 3 days of culture. These findings showed that the expression of membrane receptors depicted in Chart 2 correlated with a functional activity.

Effects of p-IL-2. The addition of p-IL-2 did not induce differentiation in the U937 cells. After 3 days of culture, there was neither increased adherence nor decreased total cell number. Concentrations of p-IL-2 as high as 57 units/ml were tested without effect. U937 cells were cultured for 3 days in m-IL-2 with and without added p-IL-2. No additional changes were induced by the p-IL-2.

Effects of IFN-γ and IFN-α. IFN-γ, and IFN-α both inhibited cell growth but did not increase cellular adherence (Table 6). Concentrations of both agents ranging from 10 to 400 units/ml were tested. The growth inhibition seen was concentration dependent. Both interferons resulted in mild increases in CR\(_{1}\) expression after 3 days in culture. IFN-γ also markedly increased the number of cells expressing Fc receptors. CR\(_{3}\) expression remained unchanged.

DISCUSSION

These results show that conditioned media from a cell line, HUT-516, derived from a patient with adult T-cell lymphoma and hypercalcemia resulted in growth inhibition, increased numbers

Table 5

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>CR(_{1})</th>
<th>CR(_{3})</th>
<th>Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22 ± 5(^a)</td>
<td>2 ± 1</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>1,25-(OH)(_2)D(_3)</td>
<td>37 ± 6(^b)</td>
<td>6 ± 1</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>m-IL-2</td>
<td>73 ± 7(^c)</td>
<td>18 ± 7</td>
<td>19 ± 5(^d)</td>
</tr>
<tr>
<td>m-IL-2 + 1,25-(OH)(_2)D(_3)</td>
<td>79 ± 6(^d)</td>
<td>61 ± 6(^d)</td>
<td>19 ± 5(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SE of 5 samples tested for each value.  
\(^b\) P < 0.01 versus control.  
\(^c\) P < 0.05 versus control.

Table 6

<table>
<thead>
<tr>
<th>IFN-γ (100 units/ml)</th>
<th>IFN-α (100 units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth (% of control)</td>
<td>Adherence (% of total cells)</td>
</tr>
<tr>
<td>Control (%)</td>
<td>100</td>
</tr>
<tr>
<td>88</td>
<td>1</td>
</tr>
<tr>
<td>74</td>
<td>2</td>
</tr>
</tbody>
</table>

DISCUSSION

These results show that conditioned media from a cell line, HUT-516, derived from a patient with adult T-cell lymphoma and hypercalcemia resulted in growth inhibition, increased numbers
of adherent cells, maturation of complement and Fc-receptor expression, and formation of multinucleated giant cells in the U937 cell model of monocytic differentiation. These maturational changes are synergistically enhanced by 1,25-(OH)₂D₃. Since there is no evidence for HTLV infection of the U937 cells cultured in HUT-516-conditioned media, one or more lymphokines secreted by the T-cells are the most probable cause of the observed changes.

IL-2, also known as T-cell growth factor, was present in the TC-CM as an essential growth factor. IL-2 is a M, 15,000 polypeptide produced by helper T-cells which stimulates the growth and activity of T-cell subpopulations (24, 29, 31). IL-2 has been shown previously to act only on T-lymphocytes (31); however, the presence of IL-2 in the TC-CM led to the consideration that it may be involved in the TC-CM induced changes. Our findings show clearly that the m-IL-2 preparation can induce differentiation in the monocytic U937 cells. The fact that the m-IL-2-induced effects on growth and adherence were significantly reduced by preincubation with the IL-2 receptor antibody indicated a specific effect of this lymphokine on the differentiation process. The presence of IL-2 receptors on U937 cells provided supporting evidence that IL-2 could affect this cell line. The IL-2 receptor has not been demonstrated on mature monocytes, and its presence on the U937 cell suggests that it retains some lymphocytic phenotype surface markers. This is not an unusual finding on malignant cell lines of hematological origin.

Since p-IL-2 did not induce these same changes, its role may be that of an important cofactor in the process of differentiation and not as the primary differentiating lymphokine. Alternatively, the α-Tac antibody may nonspecifically block the effects of the "differentiation factor" and IL-2 itself could have no function. Further purification and isolation of the various lymphokines secreted by these malignant and normal stimulated lymphocytes and their effects on the U937 used alone and in combination with IL-2 will be required to answer these questions.

The differentiation effect of the IL-2 lymphokine preparation was synergistically enhanced by the presence of vitamin D metabolites as it was with TC-CM. This synergy between vitamin D and a lymphokine is consistent with the findings of Abe et al. (1) and supports the hypothesis that both substances may be involved in the maturation of osteoclasts, a polykaryon with special functions derived from monocytic precursors (30). The m-IL-2 is obtained from pooled human leukocytes. Since conditioned media from the malignant HUT-516 cells as well as m-IL-2 induce differentiation in the U937 cells, it is evident that normal as well as malignant cells produce lymphokine(s) capable of inducing maturation.

Several investigators have shown that INF-γ induces maturation in U937 cells as evidenced by decreased cell growth, increased Fc receptor expression, change in membrane phenotype, and changes in morphological characteristics (13, 14, 22). Although we saw similar changes with cells cultured in INF-γ, the marked changes in adherence and CR3 expression did not occur; therefore, INF-γ does not seem to be the lymphokine in the HUT-516 conditioned media nor in the m-IL-2 responsible for the increased adherence and full maturation of the U937 cells.

In summary, there is evidence that 1,25-(OH)₂D₃, INF-γ, and IL-2 may induce maturational changes in U937 cells under appropriate conditions. However, the marked adherence seen with the m-IL-2 preparation cannot be accounted for solely by these agents, alone or in combination. The substance responsible for this change remains to be determined.

The clinical characteristics of patients with adult T-cell lymphoma consisting of normal serum immunoreactive parathyroid hormone values, increased turnover of bone as evidenced by bone scans, and the finding of increased osteoclast numbers present on bone biopsy (3-5, 12) all indicate that the source of the excess calcium in these patients is from resorption of bone. The cell responsible for bone resorption is the osteoclast. The hypercalcemia associated with this cancer could therefore be secondary to a stimulation of osteoclasts already present to increase bone resorption. Alternatively, it could be due to an increase in the formation of osteoclasts by promoting differentiation from their precursors presumably, monocytic-macrophages or a combination of both processes. The finding of increased numbers of osteoclasts on bone biopsy suggests that increased formation of osteoclasts is at least a component of this disorder.

Our data showing that lymphokines from stimulated normal lymphocytes induced maturation in the U937 model of monocytic differentiation supply supporting evidence that lymphokines or other substances secreted by T-cells have a regulatory role in promoting the differentiation of stem cell precursors to macrophages. The fact that multinucleated giant cells are formed after exposure to the m-IL-2 preparation, especially in the presence of 1,25-(OH)₂D₃, also supports the theory that these substances might promote osteoclast formation.

These results therefore suggest an appealing postulate for the hypercalcemia associated with adult T-cell lymphoma. The malignant T-cells secrete IL-2, INF-γ, and several other lymphokines in vivo (11, 27), and the lymphokine(s) acting in concert with other hormonal factors such as calcitriol may stimulate the differentiation of precursor cells to become osteoclasts, resulting in increased bone resorption and ultimately hypercalcemia.

ACKNOWLEDGMENTS

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REFERENCES

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