Mechanical strain inhibits expression of osteoclast differentiation factor by murine stromal cells

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Rubin, Janet, Tamara Murphy, Mark S. Nanes, and Xian Fan. Mechanical strain inhibits expression of osteoclast differentiation factor by murine stromal cells. Am J Physiol Cell Physiol 278: C1126–C1132, 2000.—Normal dynamic loading prevents bone resorption; however, the means whereby biophysical factors reduce osteoclast activity are not understood. We show here that mechanical strain (2% at 10 cycles per minute) applied to murine marrow cultures reduced 1,25(OH)2D3-stimulated formation by 50%. This was preceded by decreased expression of osteoclast differentiation factor (ODF/TRANCE). RT-PCR for ODF/TRANCE revealed that ODF/TRANCE mRNA in strained cultures was 59 ± 3% of that seen in control cultures. No significant effects on total cell count, thymidine uptake, or alkaline phosphatase activity were induced by strain. To isolate the cell targeted by strain, primary stromal cells were cultured from marrow. Mechanical strain also reduced mRNA for ODF/TRANCE to 60% of that of control in these cells. In contrast, mRNA for membrane-bound macrophage colony-stimulating factor was not significantly affected. Soluble ODF (~2 ng/ml) was able to reverse the effect of strain, returning osteoclast numbers to control. Because osteoclast formation is dependent upon ODF/TRANCE expression, strain-induced reductions in this factor may contribute to the accompanying reduction in osteoclastogenesis.

IN ITS NATURAL STATE the skeleton is loaded in a dynamic fashion. The cellular component of bone responds to changing loading patterns with an adaptive response (16, 17). For example, increasing peak compressive strains during vigorous physical activity can cause growth of bone, as seen in young athletes (4). Alternatively, the removal of load induces the resorption of the skeleton as observed when subjects are put to bed or experience microgravity conditions (24). An increase in the loading regimen, or a decrease from the previous loading situation, is sensed locally by bone cells. During adaptation to the new functional demand, remodeling of the skeleton will involve recruitment of osteoclasts from the pluripotent hematopoietic cells of the marrow. Our goal has been to understand the signals involved in the osteoclastic remodeling invoked by decreases in loading.

Both animal work and in vitro marrow cell culture have allowed biologists to define many of the processes involved in osteoclast recruitment. Although many hormones and cytokines can modulate the recruitment of osteoclasts from marrow progenitors, there are three components shown to be absolutely necessary for the basic process. The first is the marrow osteoclast progenitor itself, which must be supported by the presence of the second component, macrophage colony-stimulating factor (MCSF), throughout the lifetime of the cell (3, 9). MCSF is known to be both expressed on the surface of the stromal cells of bone, as well as secreted into the local milieu (20, 25). The third necessary component, osteoclast differentiation factor (ODF/TRANCE or OPGL), is expressed on the surface of the bone stromal cells (12, 26) and in thymus, lymph nodes, and spleen (1). This tumor necrosis factor family member, when added as a genetically engineered soluble molecule, negates the necessity for stromal support cells during osteoclast differentiation from MCSF-supported progenitor cells. We have asked whether these factors might be regulated by mechanical strain; i.e., can the application of strain limit these components?

We have previously demonstrated that application of mechanical strain, via elongation of the substrate upon which the marrow cells adhere, robustly inhibits the 1,25(OH)2D3-stimulated formation of osteoclast-like cells (19). In the work presented here, we show that ODF/TRANCE expression by bone stromal cells is inhibited by mechanical strain both in cultures of murine marrow cells and in primary stromal cell cultures, suggesting that these ODF/TRANCE-expressing cells are targets of biophysical input.

METHODS

Cell culture and analysis. Marrow cells were obtained from tibiae and femurs of 3- to 5-wk-old male C57BL/6 mice, and primary marrow cultures plated at a density of 1.1 × 106 cells/cm2 in α-MEM/10% FCS with 1,25(OH)2D3 (10 nM) in Bioflex collagen I-coated plates (Flexcell, McKeesport, PA) to stimulate formation of osteoclasts. The Institutional Animal Care and Use Committee Institutional Review Board approved all animal use. At 7 days, osteoclasts were identified by ethanol-acetone fixation and stained for tartrate-resistant acid phosphatase (TRAP). Cells that stained positive for TRAP and had three or more nuclei were counted as osteoclasts (TRAP+MNC); these cells have calcitonin receptors as shown previously (21).

To generate primary stromal cell cultures, murine marrow cells were plated in six-well plates for 30 min to separate adherent macrophages from nonadherent cells containing the
stromal elements; nonadherent cells were collected and plated at 1.4 × 10^6 cells/cm^2 in collagen-coated strain plates. Twenty-four hours later, all nonadherent cells were discarded and the remaining stromal cells cultured for 1 wk until nearly confluent (10). These stromal cells represent <5% of the initial marrow collection (7), have osteoinductive capability, and can form mineralized nodules under appropriate conditions (10). Soluble murine ODF/TRANCE (amino acids 156–315) (12) was obtained from Amgen (Thousand Oaks, CA).

Mechanical strain devices. To generate mechanical strain in primary marrow cultures, a Flexcell Bioflex instrument was used. The unit was placed inside a 37°C, 5% CO_2 incubator, and negative pressure was applied cyclically that stretched the substrate over the edge of a loading post applying a uniform and biaxial 1.8% strain across the plate surface (5). Controls were plated on similar membranes and kept in the same incubator, but not subjected to strain regimens, which are delineated for each experiment. To apply strain to primary stromal cells, either the Flexcell Bioflex (as above) or a second device, the Strainmaster (Z-Strain, Boston, MA), containing 16 30-mm wells, was used. For the Strainmaster application, silicon elastomer membranes (Dow Corning, IN) were stretched across plates and coated with collagen I (Sigma, St Louis, MO) that was sterilized under ultraviolet light. Application of 2% uniform, biaxial strain was achieved by mechanical elevation of a platen under the membrane substrate as previously described (5, 22). These devices generated similar strain responses in the primary stromal cultures and were used interchangeably.

Quantitation of thymidine uptake, cell counts, and alkaline phosphatase activity in stromal cells. To assess cell number after 5 days in culture, cells were counted by hemocytometer. For thymidine uptake, [3H]thymidine (2 µCi/well) was added on day 4 of culture; after 24 h, cells were harvested and dried onto a membrane. [3H]thymidine incorporation was assessed using a Packard 2500 TR liquid scintillation analyzer. Endogenous alkaline phosphatase activity was measured (p-nitrophenyl phosphatase activity was measured (p-nitrophenyl phosphatase substrate from Sigma) with a Sigma kit as follows: cells were washed with cold PBS and collected in 200 µl cold lysis buffer [10 mM Tris·Cl (pH 8.0), 1 mM MgCl_2, and 0.5% Triton X-100] (405 nm) reflecting phosphatase activity was normalized to protein content (nanomoles per milligram of protein per minute). A standard curve was established from 0 to 22.5 nmol/ml of p-nitrophenol in 0.02 N NaOH.

RT-PCR. Media was removed from the cell cultures (both marrow and stromal cell cultures) and cells lysed in TRIzol Reagent (BRL, Bethesda, MD). For detection of transcripts encoding ODF/TRANCE, primers were made from the gene sequence (26): the forward primer representing nt 430 was 5'-GCTAT TATGG AAGGC TCATG, and the reverse primer, at 717 nt, was 5'-CACCA TCAGC TGAAG ATAGT. Semiquantitative RT-PCR was used as reported previously, which was normalized by concurrently assaying for 18S RNA in each sample (20). PCR primers for membrane-bound MCSF (mMCSF) were used as previously reported (20). For quantification of all PCR products, forward primers were [32P]ATP end labeled using T4 kinase (GIBCO BRL) and used in a 1:5 ratio with unlabelled primers for each PCR reaction. PCR was performed on a Perkin-Elmer Thermocycler 9600 (Norwalk, CT) for 26 cycles at 30 s each (94°C, 56°C, and 72°C) and 20 cycles for 18S at 30 s each (94°C, 56°C, and 72°C). PCR products were separated on a 15% polyacrylamide gel, and densitometry of 32P signals was captured by a Molecular Dynamics PhosphorImager. Twenty-six cycles is within the linear range for generation of the ODF/TRANCE PCR product: 21 cycles generated 8.078 counts per minute; 23 gave 31.498; 25 gave 100.119; 27 gave 175.427; 29 gave 181.873; and 31 gave 226.383, as measured by densitometry of 32P signal.

Northern analysis. Total RNA made as above was chromatographed on a 1.2% formaldehyde gel and transferred to a Nitren membrane by capillary transfer. A 287-bp ODF/TRANCE probe created by PCR (nt 430–717) was random hexamer labeled with [32P]dCTP and hybridized using Quick-Hyb (Stratagene, La Jolla, CA). The 18S band on the ethidium bromide-stained gel was quantitated by densitometry, and data analyzed as density of ODF/185 in arbitrary units.

RESULTS

Strain application limits osteoclast formation in primary marrow cultures. In Fig. 1, 1.8% mechanical strain was applied with the Flexcell Bioflex loading station for 3 days (during days 2–4). As we have shown previously, mechanical strain inhibits the development of osteoclasts in murine marrow culture (19). This effect is near maximal when the strain application is during the first half of culture only, a period when osteoclast progenitors proliferate and enter the osteoclast lineage (3, 19). In this series, the strain-induced inhibition was at least 50%, compared with controls plated on similar Bioflex plates, cultured in the same incubator, but not exposed to dynamic strain.

Strain inhibits expression of ODF/TRANCE mRNA in primary marrow cultures. Primary marrow cultures were prepared the same as for osteoclast formation assays and exposed to strain during days 2–4. Total RNA was collected immediately upon cessation of strain from both strain and control cultures, and semiquantitative RT-PCR was performed for ODF/TRANCE and 18S RNA. Strain significantly inhibited the expression

![Fig. 1](http://ajpcell.physiology.org)
of ODF/TRANCE mRNA by about 40%, as shown in Fig. 2, A and B. The 18S mRNA was unaffected by the strain protocol. To confirm the RT-PCR results, Northern analysis was also performed for ODF/TRANCE mRNA on similar cultures; the inhibition of ODF/TRANCE mRNA by mechanical strain was equivalent when measured by this technique (Fig. 3).

Mechanical strain inhibited ODF/TRANCE mRNA if applied only for 24 h during day 2 (Fig. 4). Exposure to this strain regimen, i.e., 24 h of strain early in culture, also resulted in significantly decreased osteoclast formation when counted at the end of culture (38% of control). This suggests that the strain-induced decrement in ODF/TRANCE expression results in reduced potential for osteoclast formation.

To confirm that mechanical strain did not limit the proliferative capacity of the culture, both thymidine uptake and cell counting were performed on day 5, 24 h after cessation of strain. Neither cell count nor thymidine uptake was significantly altered by the 3-day strain regimen (Table 1). These parameters reflect the cells in culture at day 5, a majority of which are cells of macrophage-monocyte lineage that go on to become either macrophages or osteoclasts (7). By day 7, when the culture contains an increased proportion of stromal cells, both strain and control wells showed an equivalent distribution of fixed cells. To ascertain the stromal/osteoblastic potential of the marrow culture, i.e., those stromal cells with the potential to mineralize, alkaline phosphatase activity was measured. Under these non-mineralizing conditions, and in the presence of 1,25(OH)2D3, over three experiments alkaline phosphatase activity was not significantly changed in the presence of mechanical strain (average control alkaline phosphatase was 97 ± 17 nmol·mg−1·min−1 compared with strained plates at 93 ± 23, as shown in legend to Table 1).

Strain inhibits ODF/TRANCE expression in primary stromal cells. In the mixed primary marrow culture, those cells with the capacity to express ODF/TRANCE represent stromal cells. The effect of strain on ODF/TRANCE mRNA expression, however, could arise out of an indirect effect, for instance, on a separate cell, which might then control stromal cell expression of
ODF/TRANCE. To investigate this possibility, primary stromal cell cultures were made from marrow as described in METHODS. After 1 wk in culture, these cells reached near confluence and were subjected to 3 days of strain at 1.8%. Assessment of ODF/TRANCE mRNA expression by both RT-PCR and Northern analysis showed that strain inhibited expression by equivalent amounts to that seen in the mixed marrow cultures, by 43% (Fig. 5).

In comparison, mMCSF was unchanged by 3 days of mechanical strain in primary stromal cells (see Fig. 5A). The role of mMCSF was of interest, especially because we have previously shown that pressure, another biophysical factor, changed levels of mMCSF (18). The mMCSF was not significantly decreased by strain in experiments where ODF/TRANCE expression showed a robust inhibition due to strain.

Similar to data shown for the mixed marrow cultures (Fig. 4), we showed that decreased time exposure to strain could also inhibit ODF/TRANCE expression in primary stromal cells. Data compiled from six experiments showed that 6 h of strain given at the beginning of a 24-h period significantly reduced ODF/TRANCE mRNA expression and osteoclast formation. Primary marrow cultures were subjected to strain or not subjected to strain from days 2–4 as described in Fig. 1, and soluble ODF/TRANCE was added to the cultures at varying doses. Osteoclasts were counted after staining of cells on day 7. As shown in Fig. 6A, strain applied in this regimen inhibited osteoclast formation by 50% as expected. The addition of ~2 ng/ml soluble ODF/TRANCE increased osteoclast numbers in strained cultures to levels observed in control cultures (extrapolated from the data curve). Despite a robust dose-

Table 1. Strain does not affect proliferation or ability to make alkaline phosphatase in primary marrow cultures

<table>
<thead>
<tr>
<th>Measure</th>
<th>5 Day Control</th>
<th>5 Day Strain</th>
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<tbody>
<tr>
<td>Cell count, ×10^3</td>
<td>398 ± 11.5</td>
<td>433 ± 20.2</td>
</tr>
<tr>
<td>Thymidine uptake, ×0.01 cpm</td>
<td>497 ± 22</td>
<td>448 ± 31</td>
</tr>
<tr>
<td>Alkaline phosphatase, nmol·mg⁻¹·min⁻¹</td>
<td>127 ± 11</td>
<td>97 ± 5</td>
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Values are means ± SE of 3 wells. Primary marrow cultures were plated on collagen-coated plates and subjected to strain (or not) during days 2–4. After strain was stopped, cultures were treated with 2 μCi of [³H]thymidine per well. Twenty-four hours later (day 5), cultures were collected for assessment of cell number, [³H]thymidine uptake, and alkaline phosphatase activity. This experiment was repeated 3 times with similar nonsignificant differences; in particular, control alkaline phosphatase averaged between 3 experiments was 97 ± 17 nmol·mg⁻¹·min⁻¹, which was not significantly different from strained plates at 93 ± 23. None of the values were significantly altered by strain (P > 0.05) by Student’s t-test.

Fig. 4. A shortened strain regimen also decreases ODF/TRANCE expression and osteoclast formation. Primary marrow cultures were prepared as in Fig. 1, and test cultures exposed to strain for 24 h during day 2 only or previous regimen of 72 h (days 2–4). Total RNA was collected from 3 independent samples per condition and subjected to RT-PCR for ODF/TRANCE (top) and 18S (bottom). Both strain regimens caused reduction in ODF/TRANCE RT-PCR product. Osteoclast formation was reduced in wells exposed to early 24-h strain regimen. nd, Not done; oc #: osteoclast number.

Fig. 5. Strain inhibits ODF/TRANCE expression in primary stromal cells. A: primary stromal cells were grown for 1 wk and ± exposed to strain for 3 days after which time total RNA was collected and subjected to RT-PCR for ODF/TRANCE, membrane-bound macrophage colony-stimulating factor (mMCSF), or 18S. Data from 6 experiments, containing 2–4 independent samples for each condition after normalization to concurrent controls, are plotted on graph. Cultures not exposed to strain are the dark bars, and those exposed to strain are the lighter bars. Asterisk shows that strain inhibits ODF/TRANCE mRNA expression in stromal cultures, P = 0.0008. Decrease in mMCSF was not significant. B: cultures were generated as in A. A representative Northern blot shows ODF/TRANCE band (top) and ethidium bromide-labeled 18S band (bottom) from control (lanes 1–4) and strained (lanes 5–8) marrow cell cultures. Each lane represents an independent RNA collection made within this experiment. ODF/TRANCE/18S product in arbitrary units was 9.97 ± 0.97 for control and 6.45 ± 0.75 for strain (65 ± 1% of control). Similar results were seen in 2 repetitions.
related response of strained cultures to ODF/TRANCE, these strained cultures continued to eviscerate a significant inhibitory effect of the biomechanical force: the addition of 100 ng/ml soluble ODF/TRANCE to strained cultures resulted in only as many osteoclasts as those induced by 10 ng/ml in the control cultures. With speculation that continuous strain for 3 days during the first half of culture may have effects on other possible modulators of osteoclast recruitment, we attempted to limit the strain regimen to that necessary to cause a reduction in ODF/TRANCE mRNA expression. Because we were able to show an equivalent reduction in ODF/TRANCE with strain delivery for 6 h only (see above, 68% of control), we applied a reduced exposure to effective strain during the first half of primary marrow cell culture. As shown in Fig. 6B, strain was delivered for only 6 h/day for days 2–4. This regimen caused a significant inhibition of TRAP+MNC formation, as shown in the first set of bars. Addition of 5 ng/ml soluble ODF/TRANCE completely abrogated this effect, with osteoclast response to exogenous ODF equivalent in both control and strained plates. This experiment was repeated with similar effects.

DISCUSSION

The mechanisms by which dynamic loading of the skeleton improves the balance of bone remodeling to prevent resorption are unknown. The data presented here utilizing murine bone cells suggest that mechanical strain can inhibit the expression of a critical osteoclastogenic factor, ODF/TRANCE, and that replacement of this factor resuscitates osteoclast formation in the mechanically strained culture. These findings lead us to propose that biophysical factors can modulate recruitment of osteoclasts by limiting expression of ODF.

Strain limits osteoclast formation from marrow culture, as we have previously shown and confirm here (19). Importantly, the application of strain can be limited to a small window of time, even as short as 24 h during days 2–4, and is not effective when applied out of this time window. During the time of efficacious application, osteoclast progenitors divide under the influence of MCSF (3), and a small percentage enter the osteoclast lineage (7). Fusion of osteoclast progenitors proceeds during the second half of culture (3, 6), encompassing events that appear to be immune to the influence of strain. With this work we have shown that the mRNA for ODF/TRANCE, a critical factor displayed by indispensable stromal cells within the mixed marrow culture, is significantly downregulated by strain during the susceptible time window.

It is important to determine whether the decrease in ODF/TRANCE mRNA in the mixed marrow culture is due to decreases in the cell population expressing ODF/TRANCE, or a direct effect of strain on ODF/TRANCE gene expression. To address this question, we ruled out that 3 days of strain exposure caused a general inhibition of cell proliferation. Most of the cells present and proliferating at this time in culture are cells of macrophage lineage (7), and we may not be able to measure small changes in the osteoprogenitor population. However, we also showed that alkaline phosphatase, presumably expressed by cells capable of osteoblastic differentiation, was not altered by strain during the effective strain regimen. We have previously shown
that this regimen of strain does not affect numbers of alkaline phosphatase expressing cells at the end of culture (19). Taken together, these results suggest that strain may have a direct effect on ODF/TRANCE gene expression, rather than modulating differentiation of the cell that displays this critical molecule. The inhibitory effect of strain on ODF/TRANCE expression appears to be the major mechanism by which strain limits osteoclastogenesis. Strain delivered for 6 h/day was sufficient to reduce ODF/TRANCE expression, as shown in RESULTS, and to inhibit osteoclastogenesis, as shown in Fig. 6B. Application of soluble ODF/TRANCE completely abrogated the strain inhibition of osteoclast formation. As shown in Fig. 6A, however, effects of strain on ODF/TRANCE expression may not be the only mechanism by which strain can limit osteoclastogenesis, because the ability of soluble ODF/TRANCE to generate osteoclasts was blunted in the strained cultures. This blunted response might arise from effects of continuous strain on other factors besides ODF/TRANCE expression. For instance, there is a possibility that strain could limit the ability of osteoclast progenitor cells to respond to the ODF/TRANCE molecule, or responses to other chemokine modulators such as interleukins 6 or 11 might be affected. However, although we do not fully understand the extent of strain effects on all the components present in the mixed marrow culture, our work supports the hypothesis that strain suppression of ODF/TRANCE is responsible for a large part of the suppressive biomechanical response.

To further confirm that stromal cells were the targets of strain, primary stromal cell cultures were made from murine marrow. These cells are able to differentiate under appropriate conditions and form mineralized nodules (2, 11, 15). As we have shown here, these osteoprogenitor cells also express ODF/TRANCE in the presence of 1,25(OH)2D3. Primary stromal cells had a nearly identical decrease in ODF/TRANCE mRNA expression after exposure to 3 days of mechanical strain as demonstrated by both RT-PCR and Northern analysis. We did not observe increases in alkaline phosphatase in the strained cultures, as was seen in human osteoblast-like cells strained previously (8). Several differences exist between that investigation and ours: the strain system utilized in that work generates a nonuniform strain with significant shear stress (5), and the cells studied were more differentiated than the stromal cells extant during the first several days in the marrow cultures used in our work. Our data indicate that strain, in the absence of shear, has a direct effect on stromal bone cells, and suggest that strain may directly regulate the ODF/TRANCE gene itself.

Another molecule of critical importance to the development of osteoclasts, and, as well, displayed in a paracrine fashion by stromal cells, is MCSF. Indeed, we have shown that hydrostatic pressure, which also limits osteoclastogenesis, decreases the mRNA for the membrane-bound form of MCSF (18), and it has been suggested that ovariectomy increases the expression of this MCSF mRNA species in murine bone marrow cells (13). In studies presented here, effects of strain on mMCSF mRNA were not statistically significant. Differences between the responses of biological tissues to varying physical factors are poorly understood. It is likely that cellular effects due to sustained increases in hydrostatic pressure, which do not distort the cell membrane, are dissimilar to those resulting from strain. Thus, although the level of mMCSF displayed by stromal cells must support osteoclast recruitment to bone, regulation of MCSF levels does not appear to underwrite the robust inhibition of osteoclast formation induced by strain.

Other investigators have postulated that the effects of mechanical loading are transmitted to bone cells largely through the fluid flow propagated by the load. Smalt et al. (23) were not able to measure alterations in the production of nitric oxide and prostaglandin E2 from primary rat calvarial cells subjected to 500–5,000 microstrain for 20 min. In comparison, shear stress of <5 dyn/cm2 caused immediate increases in these products. Although our system is entirely different in that strain is delivered for at least 6 h, our target cell is less differentiated, and different endpoints (osteoclastogenesis and ODF/TRANCE expression) were measured, two comparative points must be made. Neither strain system used in our work imposes biologically relevant fluid stress, as reviewed previously (5, 19). Both strain instruments delivered strain at ~2% (20,000 microstrain), which is higher than that delivered during 20 min to differentiated calvarial cells.

Although the loads that are delivered to cells in a tissue culture plate are in excess of those likely seen in vivo, the model, as any in vitro model, serves to render clues as to what might be targets of mechanical load. Loads sufficient to maintain skeletal mass appear to be within the 500–5,000 microstrain ranges (14), although this measurement pertains to the hard tissue only. The loads that we have applied may be fivefold higher than those experienced in vivo; however, the theoretically smaller physiological strains are active over months, rather than days, during the remodeling cycle in the skeleton. Thus, although our model may not duplicate the exact magnitude and temporal process in the in vivo skeleton, it represents one of the most powerful ways to understand those processes that are subject to regulation by strain.

Dynamic loading of the skeleton imposes a limitation on bone resorption. Cessation of exercise leads to recruitment of osteoclasts and bone resorption as the skeleton adapts to a new loading regimen. At its most positive, the effect of loading the skeleton will cause a positive adaptation, i.e., an increase in bone formation. Our in vitro results suggest that one mechanism by which mechanical load prevents osteoclast recruitment locally is by modulating the level of the paracrine ODF/TRANCE necessary for osteoclast differentiation, positively shifting the balance of the coupling between formation and resorption. Thus one of the biophysical signals that can be recognized by relevant cells is strain. The signaling pathway by which deformation of stromal cells results in changes in gene expression is unknown, but this should be an exciting area of research in the future.
MECHANICAL STRAIN INHIBITS ODF/TRANCE IN BONE STROMAL CELLS

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-42360 and a Veterans Affairs Merit Review.

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Received 8 July 1999; accepted in final form 20 December 1999.

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