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Opposing Effects of Tyrosine Kinase Inhibitors on Mineralization of Normal and Tumor Bone Cells

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Abstract Induction of matrix maturation and mineralization in calcified tissues is important for patients with primary bone tumors and other bone deficiencies, e.g., osteoporosis. For the former it signifies a better prognosis in osteosarcoma, and for the latter it might improve bone remodeling. In the present study we exposed osteosarcoma cells (Saos2), normal bone cells, and marrow stroma to two different tyrosine kinase (TK) inhibitors: AG-555 and AG-1478. These tyrphostins differ in their effect on signal transduction downstream to the TK receptor (RTK): AG-1478 inhibits src family TKs whereas AG-555 inhibits nuclear TKs. We found that both tyrphostins at 50 μM increased specific alkaline phosphatase (ALP) activity in Saos2 cells. AG-555 abrogated mineralization whereas AG-1478 increased it. Similarly, in human bone-derived cell cultures the same dose of tyrphostins had an opposing effect on mineralization but, in contrast to AG-555, AG-1478 positively selected cells with ALP activity. These tyrphostins also differed in their effect on rat marrow stromal cells. AG-555 decreased cell counts unselectively, whereas the decreased cell counts by AG-1478 resulted in selection of osteoprogenitor cells as indicated by a concordant increase in specific ALP activity. The effect of a lower dose of AG-1478, 5 μM, on the increase in mineralization exceeded its own efficiency in selecting cells with specific ALP activity. Our results indicate that AG-1478 selects and preserves the osteoblastic phenotype, at doses moderately higher than those required to induce mineralization, and substantially higher than the doses required for RTK inhibition. Identification of downstream molecular targets for AG-1478, in marrow stromal cells, might prove useful in designing more selective drugs, capable of separating proliferative from differentiation-inducing activities. J. Cell. Biochem. 65:420–429.

Key words: osteosarcoma; osteoprogenitors; tyrphostins; marrow-stroma; quinazoline; benzylidene-malononitrile; cell proliferation

Natural tyrosine phosphorylation inhibitors, e.g., genistein, have been advocated as useful agents in the prevention of cancer [Massina et al., 1994], based on epidemiological observations. One biochemical rationale for efficacy of such agents as anticancer drugs is related to their ability to block tyrosine kinase (TK) [Akiyama et al., 1987], since cell proliferation depends on signal transduction resulting from TK receptor (RTK) activation [Pawson, 1992]. Thus TK became targets for drug design resulting in generation of synthetic TK inhibitors (tyrphostins) of different specificities [Levitzki and Gazit, 1995]. Tyrphostins act on TK by substrate inhibition, and by competing for an additional target that is commonly sought by many tyrphostins, i.e., the ATP binding sites within the TK [Ulrich and Schlessinger, 1990]. ATP binding is a conserved characteristic of TKs, thus tyrphostin competition with ATP should presumably keep adenine nucleotides away from their binding sites and freely available at the intra- and perhaps extra-cellular milieu. A putative increase in free ATP may provide a linkage between tyrphostins and skeletal tumors since it was shown that free ATP enhances endochondral mineralization [Blumenthal et al., 1977]. Hatori et al. [1995] have shown that growth plate chondrocytes degrade ATP and ADP, thereby inducing mineralization. It is, therefore, expected that tyrphostins might have at least two separate effects on the mechanism of growth arrest in normal and malignant bone cells. One is by blocking signal transduction via inhibition of RTK activation. The other is a yet unproved effect on the pool of free adenine nucleotides, which in turn affects mineralization, by inhibiting mitochondrial oxidative phosphorylation, as suggested by Hatori

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et al. [1995]. Since mineralization is tissue specific for skeletal cells, the relevant target cells for this effect of tyrphostins are osteosarcoma cells. This is relevant to the observation of Huvos et al. [1977] that the best indicator for success of chemotherapy in osteosarcoma is the extent of necrosis and calcification foci at the tumor site. According to the above rationale, tyrphostins should induce or increase calcification, making them commendable drugs for growth suppression of osteosarcoma.

In the present study we examined two tyrphostins, AG-1478 and AG-555, which differ by their inhibitory effect on TKs downstream to the RTK (epidermal growth factor receptor, EGFR), and also by the dose required for EGFR inhibition [Levitzki and Gazit, 1995]. We tested their effect on human osteosarcoma and normal bone cells in culture, and on proliferation and differentiation. In addition, we tested the effect of these tyrphostins on a rat bone marrow stroma culture-system adapted for drug testing [Klein et al., 1993a]. Our results show that AG-1478 (quinazoline derivative) effectively suppressed growth of Saos2 osteosarcoma cells, increased mineralization, and selectively preserved the osteoblastic phenotype. Conversely, AG-555 (benzylidene malononitrile derivative) killed cells without distinguishing between cell types, and decreased mineralization.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, dexamethasone, ascorbate, and β-glycerophosphate were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Grand Island Biological Company (Grand Island, NY). Tyrphostins were obtained from Prof. A Levitzki.

Stromal Cell Culture

Bone marrow cell suspensions obtained from femurs and tibiae of female Sabra rats, weighing 60–80 g, were seeded in 25 cm^2 flasks, 10^8 cells/flask as in Maniatopoulos et al. [1988]. Stromal cells were obtained by removing the non-adherent hematopoietic cells during the first 10 days of culture. The remaining adherent stromal cells were propagated in the same maintenance medium that consisted of DMEM (Dulbecco modified Eagle's medium) suplemented with 15% FCS and antibiotics. The cells were incubated at 37°C in a humid 9% CO_2 atmosphere. For the experimental cultures, cells were removed 7 days later by trypsinization and plated in 96-well plates, 3,000 cells/well, and grown in ordinary medium or osteoprogenitor cell (OPC) stimulation medium. This consisted of maintenance (ordinary) medium containing 10^{-8} M dexamethasone (DEX), 50 μg/ml ascorbate, and 10 mM β-glycerophosphate, designated DEX-medium.

The human osteosarcoma line, Saos2, was maintained in 25 cm^2 culture flasks in DMEM with 5–10% FCS, seeded at 2,000–5,000 cells/cm^2 and transferred by trypsinization every 3–5 days.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. Day 11 of dexamethasone stimulation was set for ALP assay and cell counting [Klein et al., 1993a]. The medium was removed and the cells were washed twice in situ with 0.2 ml TBS (50 mM Tris, 150 mM NaCl, pH 7.6). ALP substrate, pNPP (p-nitrophenylphosphate) in TBS, 1.33 mg/ml, was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min and optical density of the hydrolysed pNPP was measured in a multichannel optical densitometer at 405 nm wavelength. This protocol enables long incubation times as hydrolysis is 30 times slower than the usual protocol [Klein et al., 1993b]. ALP specific activity was calculated as nMol/90 min/50,000 cells.

Quantitative Cell Staining

Cells at the lower half of the 96-well plates were stained using the methylene blue (MB) method. Cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with D H_2O, and air-dried over night. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH) 0.2 ml/well, was added to the cells for 2 min and rinsed with tapwater. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed with water, and air-dried. The MB was eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 min. O.D. of the eluted MB was measured at 620 nm.

Measurement of In Vitro Precipitated Calcium

After 3 weeks in culture, OPC stimulation medium as opposed to ordinary medium in-

duces cell mediated calcifications in uninhibited cultures [Klein et al., 1993b]. To measure the precipitates, plates were washed twice with TBS and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by the arsenazo-III metallochromic method adapted to microplates [Klein et al., 1995] against samples of known calcium concentrations. Mineralization is expressed as µg calcium/well.

RESULTS

Effect of AG-555 and AG-1478 on Saos2 Cell Proliferation, ALP Activity, and Mineralization

Figure 1A shows that AG-1478 suppresses Saos2 cell growth at 5 and 10 µM more efficiently than does AG-555. Both tyrphostins completely suppressed cell growth at 50 µM. This concentration was used to test the effect of these tyrphostins on ALP activity and mineralization. Their effect on these parameters was tested under conditions that simulate osteogenic induction in normal rat osteoprogenitors (with dexamethasone, DEX). Figure 2 shows the effect of these tyrphostins on cell growth and specific ALP activity with and without DEX stimulation. DEX was added on day 4 and tyrphostins on day 6 after seeding. Note that in the absence of DEX, tyrphostins were added to confluent cultures; therefore, growth arrest is probably not a result of the anti-proliferative effect of tyrphostins. Yet AG-1478 caused a 2/3 reduction in cell number in addition to the growth arrest (Fig. 2A). DEX had a similar effect on proliferation but had no additive effect with AG-1478 (Fig. 2B). AG-555, at 50 µM, only arrested cell growth (Fig. 2A), but combined with DEX cell counts diminished by an additive effect. Both tyrphostins induced a slight increase in specific ALP activity (Fig. 2C) which was abolished when tyrphostin treatment was combined with DEX (Fig. 2D). Figure 3 shows the effect of tyrphostins on mineralization using the same protocols. AG-1478 significantly increased mineralization only in the absence of DEX whereas AG-555 inhibited it with and without DEX. Thus these tyrphostins affect Saos2-mediated mineralization differentially in the absence of DEX stimulation but both inhibit mineralization in DEX-stimulated cultures. These results indicate that the differential effect of these tyrphostins on Saos2 cell-mediated mineralization is not linked to ALP activity.

Effect of AG-555 and AG-1478 on Marrow Stroma-Derived Osteoprogenitors (OPC)

DEX-stimulated rat stromal cells were used to test the response of OPC to tyrphostins. Figure 4 shows the response of OPC to 50 µM of tyrphostin, added to the cultures between day 3 and 7 of DEX stimulation. AG-555 slightly increased specific ALP activity (Fig. 4A, left) proportionally less than the decrease in cell count (Fig. 4B, left), therefore it cannot be interpreted as a selection of ALP-expressing cells. However, the 40% decrease in cell count was accompanied by only 7% decrease in mineralization contrary to the effect on Saos2 tumor cells, where no decrease in cell count was seen (Fig. 2B) but a much higher decrease in mineralization (Fig. 3). AG-555 in the presence of DEX suppressed growth of normal rat marrow stromal cells more effectively than growth of Saos2 osteosarcoma cells.

AG-1478 showed a 3-fold decreased cell count (Fig. 4B, right) vis-à-vis a 4-fold increase in specific ALP activity and a 25% increase in mineralization (Fig. 4A, right). This indicates that AG-1478 inhibits growth of non-OPC, and that OPC exhibit substantial resistance to AG1478, which was also observed in surviving Saos2 cells (Figs. 2B and 3). To compare the response of human normal bone-derived cells to these tyrphostins with the response of rat stromal cells, we examined human bone-derived cells. Figure 5 shows the response to a high tyrphostin dose (50 µM) of DEX-stimulated normal bone cells. Unlike its effect on rat stroma, AG-555 decreased specific ALP activity in normal human bone cells by 57%, mineralization by 70% (Fig. 5A, left), and total cell count by only 17% (Fig. 5B, left). AG-1478 had an opposite effect; specific ALP activity in the AG-1478-treated cells was ninefold higher than in the controls, and was accompanied by an 86% increase in mineralization (Fig. 5A, right). AG-1478 induced a 95% decrease in cell count in accord with the ninefold increase in specific ALP activity. Thus, AG-1478 preserves the osteoblastic phenotype and stimulates its mineralization similar to the response seen in the normal rat stromal cells (Fig. 4), whereas in the transformed cells (Saos2) this phenotype preservation is incomplete (retaining specific ALP on day 9 but not on day 11, Fig. 2D) under AG-1478 with DEX stimulation, and mineraliza-

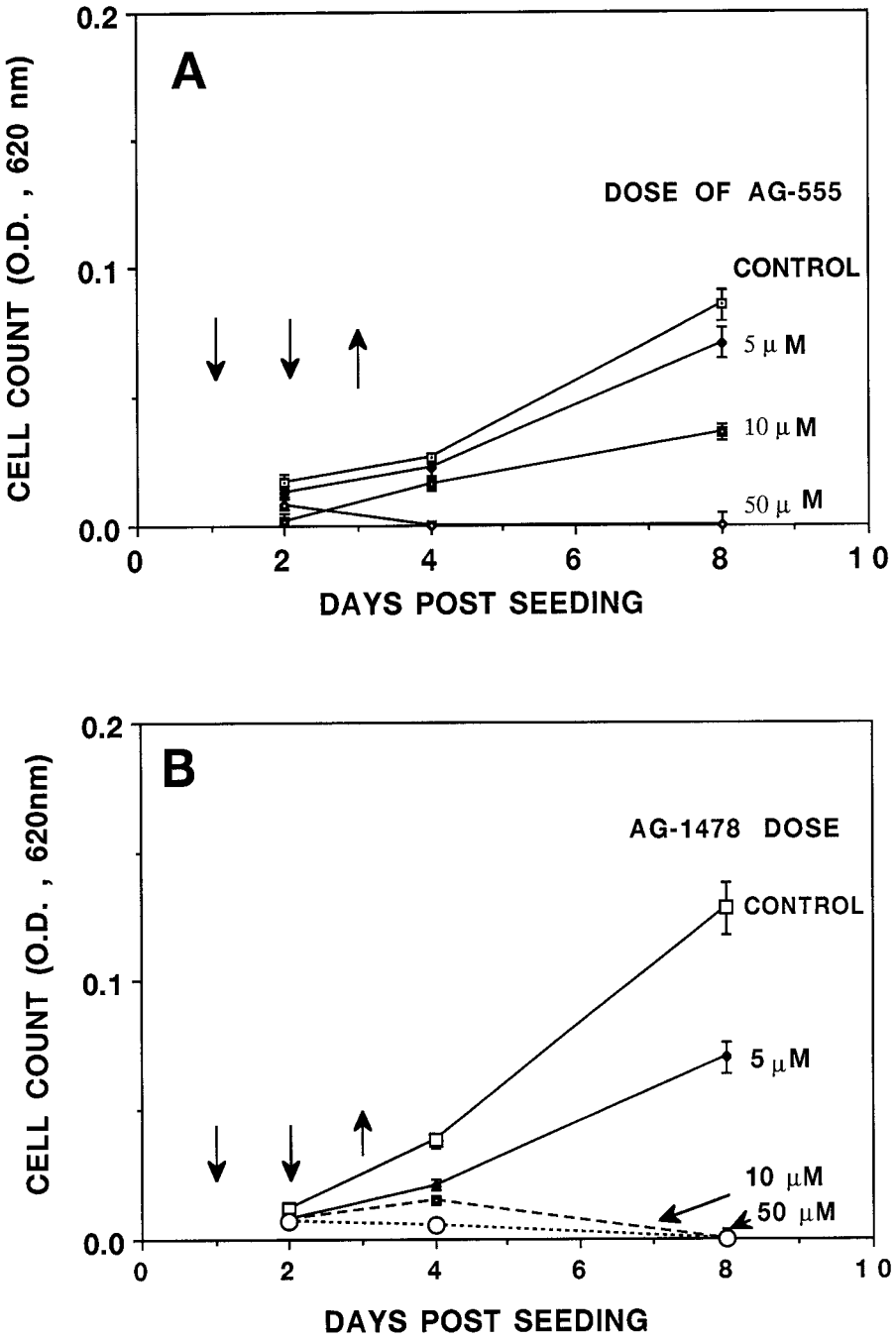

Fig. 1. Dose response of Saos2 cell growth to tyrphostins after a 48-h exposure: Saos2 cells, 2,000 cells/well, were seeded on day 0 in microtiter plates. Tyrphostins, at indicated concentrations, were applied to the cultures on days 1 and 2 (downward arrows) and removed on day 3 (upward arrow). Cell counts were performed on days 2, 4, and 8. Each point represents the mean ± SEM, n = 15.

tion takes place only in the absence of DEX (Fig. 3).

Dose Response of Rat Stroma to AG-1478

To determine the concentration of AG-1478 that selects the OPC phenotype in DEX-stimulated normal rat stroma, we added tyrphostins to the cultures between day 3 and 7 of DEX stimulation. Figure 6 shows that even 5 μM of AG-1478 could select cells with high ALP activity (Fig. 6A), but selection efficiency was much less than with 50 μM as suggested by the recip-

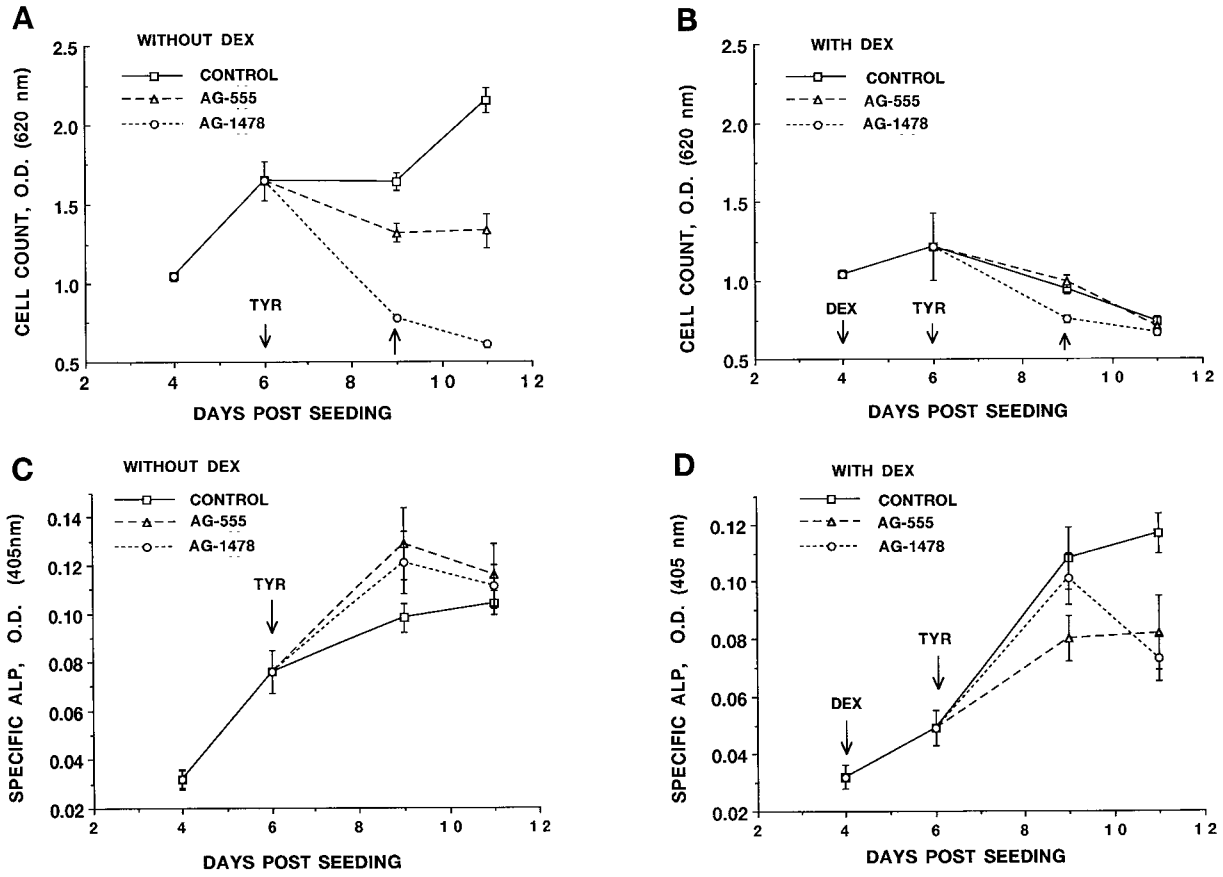

Fig. 2. Effect of a 72-h exposure to high dose tyrphostins on Saos2 cell growth and ALP, in the presence or absence of DEX stimulation. Saos2 cells, 1,000/well, were seeded on day 0 and cultured in 5% FCS to retard confluence. Tyrphostins, 50 µM AG-555 or AG-1478, were added on day 6 and removed 72 h later. DEX-stimulated cultures were grown in 10 nM DEX from day 4. Cell counts and ALP assays were performed on days 4 and 6, prior to drug addition, and on days 9 and 11. Each point represents the mean ± SEM, n = 15.

rocally coordinate growth arrest (Fig. 6B). Figure 7A shows mineralization on day 21 of DEX stimulation in response to AG-1478 doses identical to those described in Figure 6. The increased mineralization induced by 5.0 µM was not significantly different from that induced by 50 µM AG-1478. This indicates that in addition to selection of ALP-expressing cells, and perhaps by a separate mechanism, AG-1478 augments mineralization by the OPC regardless of the presence of non-OPC.

Figure 7B shows the level of mineralization in cultures exposed to similar concentrations of AG-555 between days 7 and 14, after AG-1478 was removed. At low concentrations, AG-555 inhibited mineralization, antagonizing the positive effect of AG-1478. At 50 mM, AG-555 did not inhibit mineralization (Fig. 4A,B, left). This might indicate the presence of an additional effect of high dose AG-1478 on mineralization, which opposed the effect of AG-555 (Fig. 7B).

DISCUSSION

The two tyrphostins used in this study are known to be highly effective blockers of signal transduction initiated in response to EGF receptor (EGFR) activation. AG-1478 can block EGFR auto phosphorylation at the nM range [Osherov and Levitzki, 1994]. In the present study, we found a substantial recovery of Saos2 cells from short-term (48 h) exposure to either one of these drugs at concentrations up to 10 µM, which is consistent with a reversible inhibition of TK activity. AG-1478 is also known to inhibit the phosphorylation of src family proto-oncogenes, downstream of the EGFR [Osherov et al., 1995]. This was observed at concentrations higher than those required to inhibit EGFR.

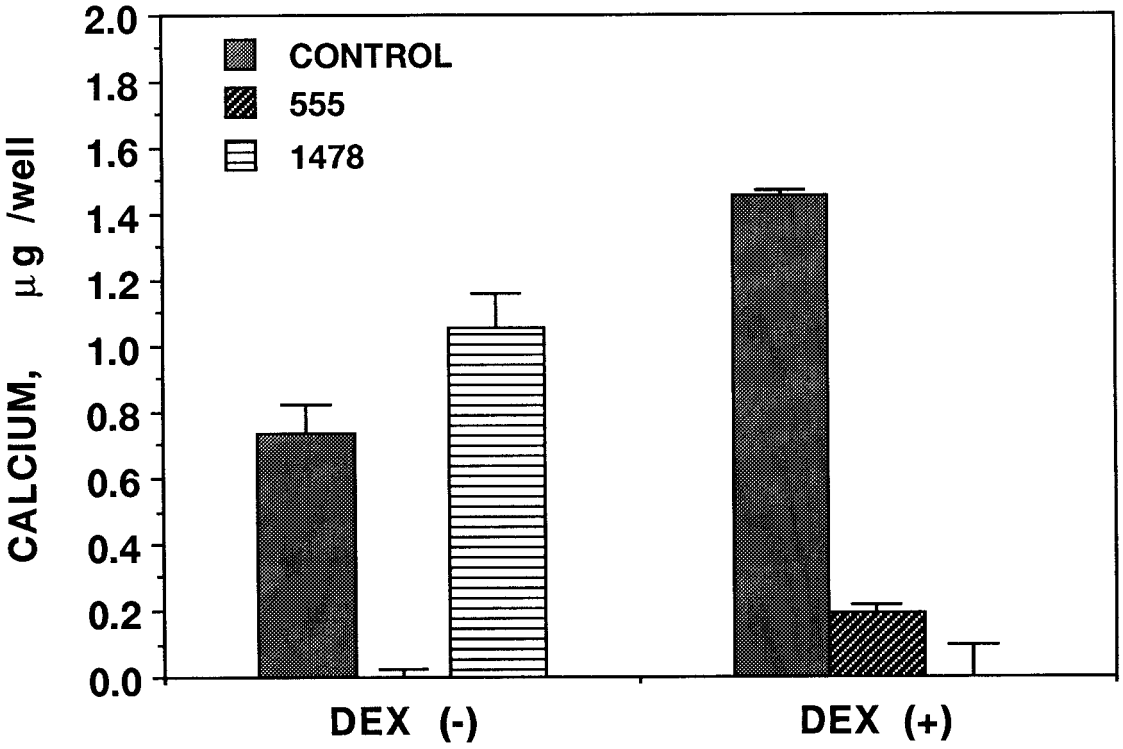

Fig. 3. Effect of a 72-h exposure to high tyrphostin dose on Saos2 cell-mediated mineralization. Cultures were grown and treated by the same protocols as described in the legend of Figure 2, except that on day 21 acid-soluble calcium was measured.

Ben-Bassat et al. [1995] have shown that a high dose AG-555, 50 µM, can arrest keratinocyte proliferation at the S phase of the cell cycle, which suggested that AG-555 too, has a downstream effect beyond EGFR inhibition. The difference in recovery level shown in the present study is consistent with the differential effects of these drugs each on their own downstream target, tyrosine kinases, and/or other targets. When tyrphostins were applied to Saos2 cells after a 6-day-long propagation, at a higher cell density, AG-555, at 50 µM, arrested cell growth but spared many of the cells, unlike 50 µM AG-1478, which drastically reduced cell counts. Interestingly, surviving cells under each of these 2 drugs showed a higher specific ALP activity than controls, but, on the other hand, AG-1478 resulted in a higher mineralization than controls, whereas AG-555 inhibited mineralization. This differential effect might suggest that cellular control of mineralization is associated with osteoblastic cell death, perhaps by apoptosis [Kim, 1995]. In addition, mineralization can be inhibited differentially from the inhibition of ALP activity. It thus follows that the opposing effects of these two drugs are more specific to the mechanism involved in mineralization control than to other aspects of osteogenesis. The main characteristics shown by AG-1478 in culture are reminiscent of an in vivo observation in patients under chemotherapy for osteosarcoma, in which the success of chemotherapy is best judged by the degree of necrosis and calcification within the tumor site [reviewed by Davis et al., 1994].

Dexamethasone showed some similarity to AG-1478 in affecting Saos2 osteosarcoma cells. The similar effects on proliferation are not well understood, as DEX augments EGFR transcription [Hudson et al., 1989], which is expected to increase EGFR activity, being rather antagonistic to the effect of AG-1478. The similarity of AG-1478 to DEX effects on mineralization is uncertain as well, but it can be hypothesized as follows: We have shown that DEX modulates the mitochondrial membrane potential (MtMP) [Klein et al., 1993c] and that the MtMP modulation pattern is in accord with cell-mediated mineralization [Klein et al., 1996a]. In addition, mineralization can be associated with un-

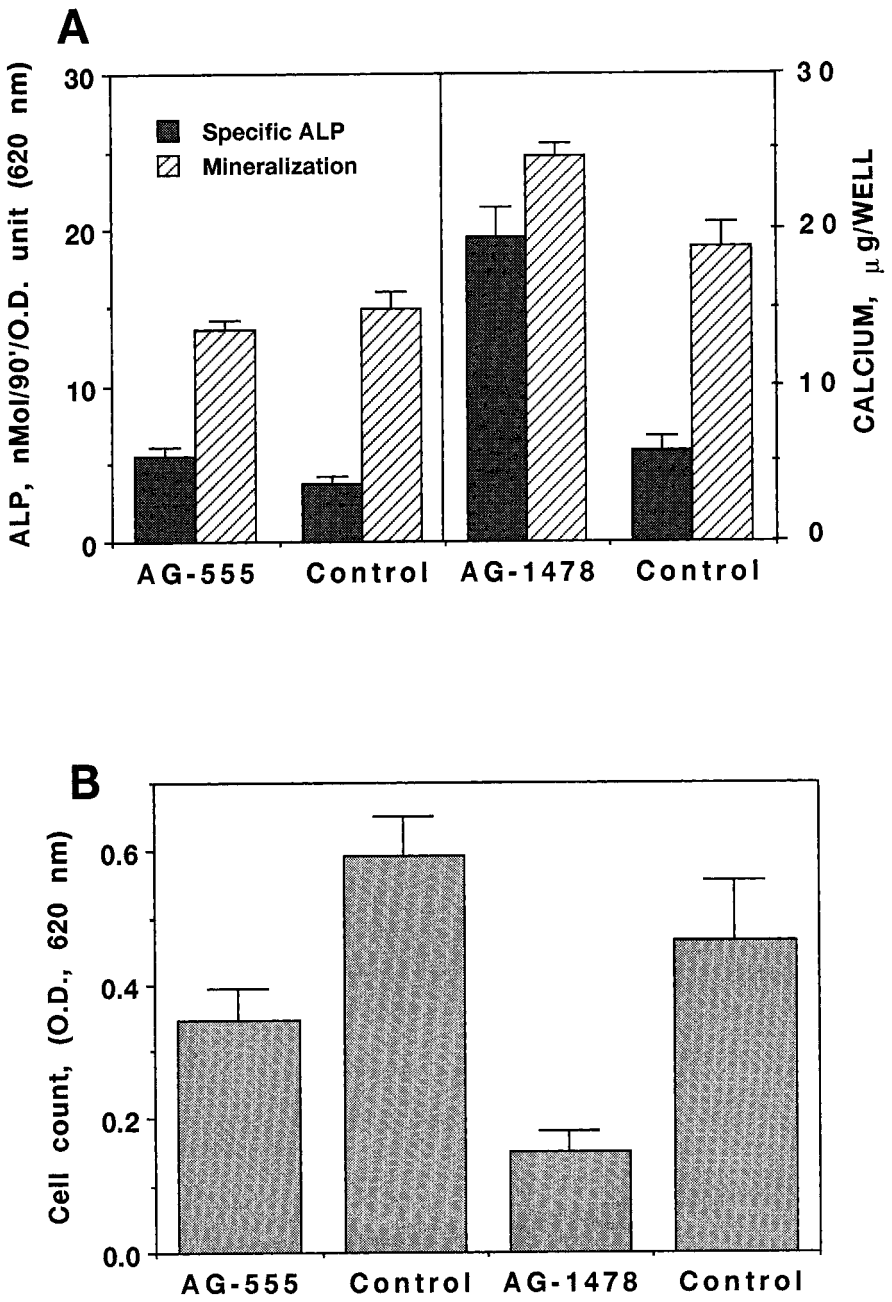

Fig. 4. Effect of high-dose tyrphostins on stromal cell-derived OPC. Stromal cells, 3,000/well, were seeded on day (−)3 and DEX-stimulated from day 0. Tyrphostins, 50 µM AG-555 or AG-1478, were applied to the cultures on day 3 and removed on day 7. **A:** Specific ALP activity on day 11 and µg calcium/well on day 21. **B:** Cell counts on day 11. Results are expressed as mean ± SEM, n = 20.

coupling of oxidative phosphorylation [Klein et al., 1996b], which may link the effect of DEX to mineralization. Since AG-1478, like other tyrphostins, competes for ATP binding sites, it could interfere with FoF1 ATP synthase, or carrier, at the mitochondria. Thus, AG-1478 may increase mineralization by uncoupling of oxidative phosphorylation, but we have no indication as to whether AG-1478 causes it by a generalized release of endogenous ATP or by blocking adenine nucleotide binding at the mitochondrial ATP synthase. Based on these considerations, it is possible that both DEX and AG-1478 are targeting the same mitochondrial function, i.e., interference with MtMP, which may explain their similar effect on mineraliza-

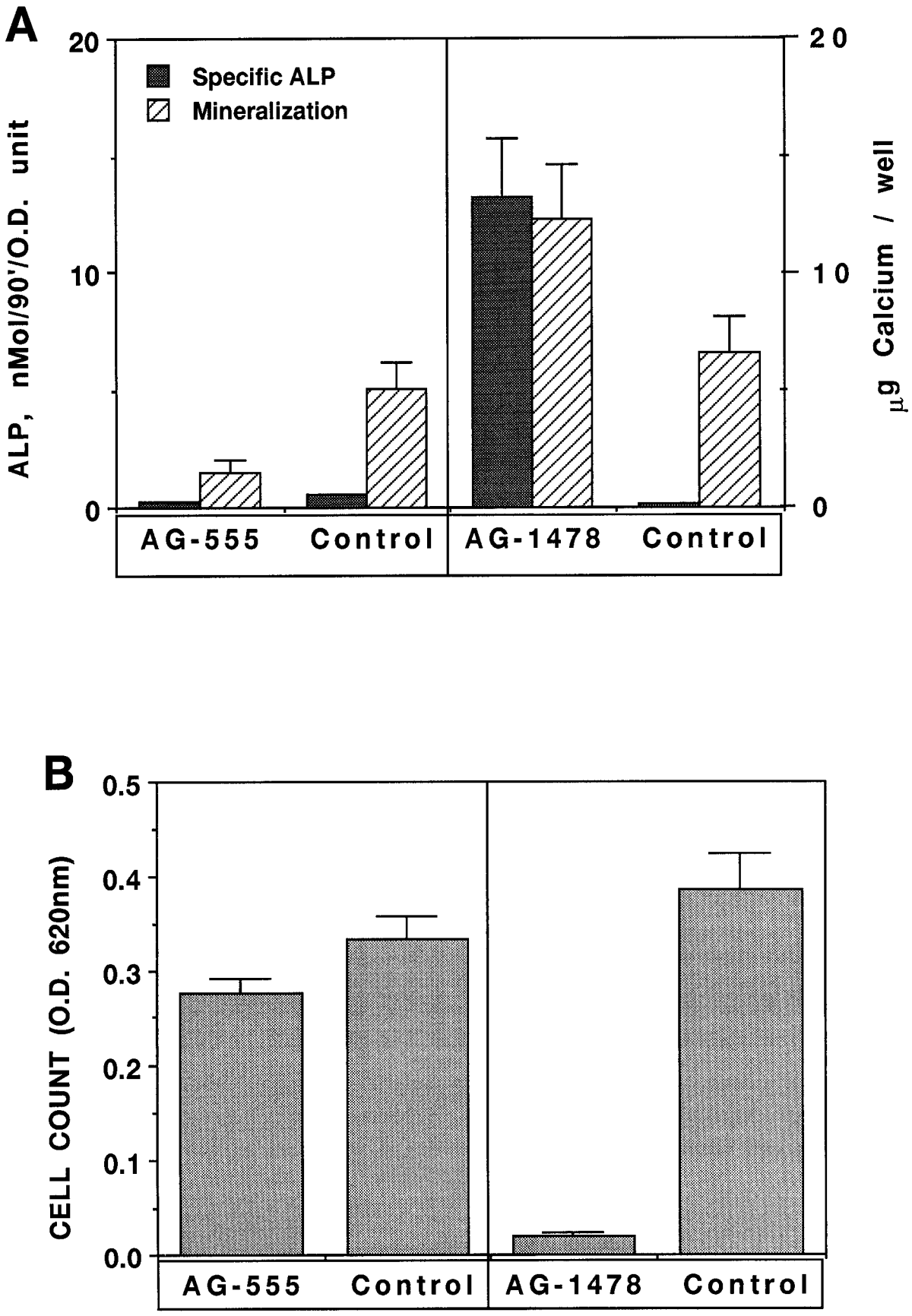

Fig. 5. Effect of high-dose tyrphostins on human bone–derived cells. Human bone–derived cells were seeded on day (−)3 and treated as described in the legend of Figure 4.

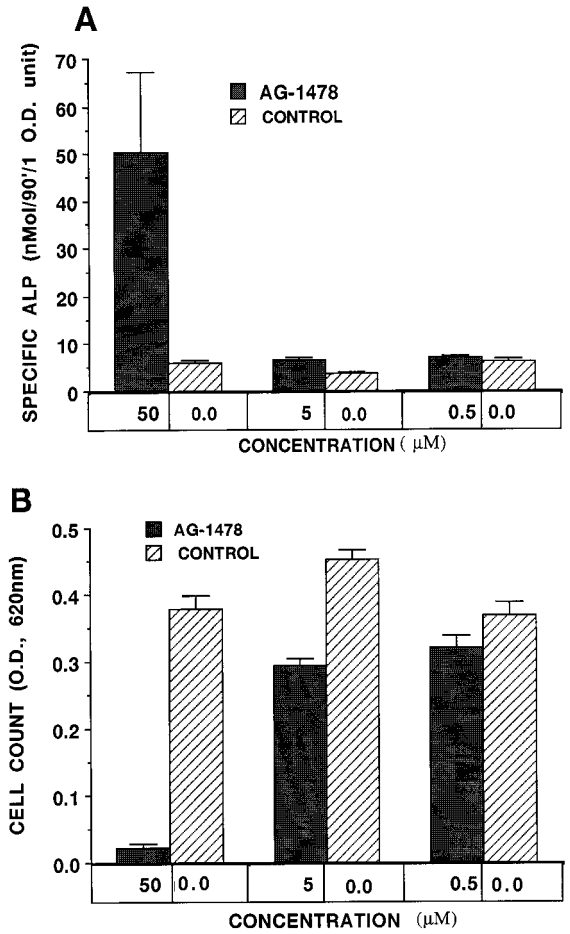

Fig. 6. Dose response of stromal cells to AG-1478, effect on ALP, and cell count. Rat stromal cells were exposed to indicated doses of AG-1478 on days 3–7 of DEX stimulation. **A:** Specific ALP activity; **B:** cell count. Results are expressed as mean ± SEM, n = 20.

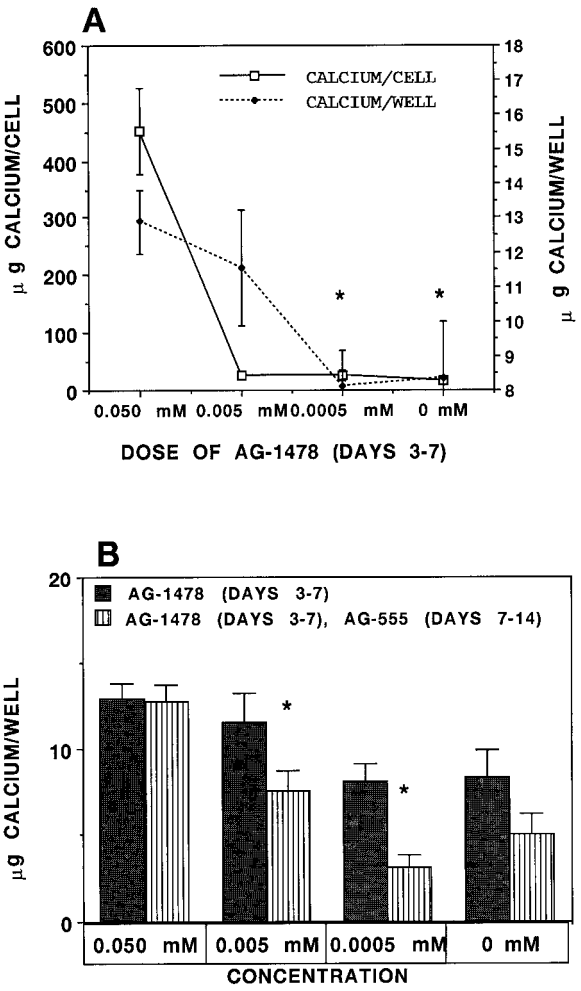

Fig. 7. Dose response, of stromal cell mineralization, to AG-1478 and the antagonizing effect of AG-555. Stromal cells were exposed to several doses of AG-1478 between days 3–7 of DEX stimulation. **A:** Day 21 mineralization, per cell and per well. Asterisks indicate non-significantly increased mineralization. **B:** Day 21 mineralization in cultures exposed to AG-1478 on days 3–7, which was then replaced by equivalent concentrations of AG-555 from day 7 to 14. The results are compared with the values obtained from controls, which were exposed only to AG-1478 on days 3–7, n = 20. Asterisks indicate a significant decrease in mineralization.

tion. Interestingly, genistein was considered to be an uncoupler of oxidative phosphorylation [Lundh and Lundgren, 1991]. It should be noted that the positive effect of tyrphostins on bone, which was shown by others [Yoneda et al., 1993; Hall et al., 1994], was related to inhibition of the TK of $pp60^{c\text{-}src}$ in osteoclasts, thus inhibiting bone resorption. This activity is irrelevant to our studies as we failed to identify giant cells in the cultures. Thus, our system represents osteogenic rather than osteolytic activity.

Osteoprogenitor cells (OPC) constitute a small fraction of the marrow stroma. High-dose AG-555 applied to DEX-stimulated stromal cells at confluence decreased cell counts by 40% with no selection of OPC. In contrast, AG-1478 decreased cell counts twice as much but preserved the osteoblastic phenotype and slightly increased mineralization.

High-dose AG-555 effect on cell counts of human bone–derived cells was minimal perhaps because most of them were at an advanced differentiation stage. In contrast, high-dose AG-1478 killed most of the normal human bone cells and preserved a population with high ALP activity and caused increased mineralization. Preservation of normal human bone cells relative to the killing of osteosarcoma cell line by high dose of AG-555 may deserve further investigation of tyrphostins as anti-osteosarcoma chemotherapy. AG-555 combination with lower

doses of AG-1478 may confer increased mineralization to the osteosarcoma (a characteristic of favorable response to chemotherapy) [Davis et al., 1994] and still be tolerated by normal bone. In DEX-stimulated rat stromal cells, a medium dose (5 μM), but not a small dose (0.5 μM), of AG-1478 killed 1/3 of the cells. The OPC were included among the surviving cells and mineralization per well was not significantly different from that under high dose (50 μM). The same medium-dose of AG-1478 can select mineralizing cells among DEX-unstimulated cells almost as efficiently as among DEX-stimulated cells (not shown). It is, thus, clear that AG-1478, unlike AG-555, can select OPC among stromal cells, and induce at least one function of terminal differentiation (mineralization) and also induce cell death, perhaps apoptosis. It is not clear yet if in these OPC the mineralization, the cell death, and uncoupling of oxidative phosphorylation are all different aspects of the same phenomenon, and whether they can be uncoupled.

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