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Phosphatidylinositol 3-Kinase Coordinately Activates the MEK/ERK and AKT/NFκB Pathways to Maintain Osteoclast Survival

Anne Gingery,[1] Elizabeth Bradley,[1] Aubie Shaw,[1] and Merry Jo Oursler[1,2]*

[1]Department of Biochemistry and Molecular Biology, University of Minnesota, Duluth, Minnesota 55812
[2]Departments Biology, and Medical Microbiology and Immunology, University of Minnesota, Duluth, Minnesota 55812

Abstract We have examined highly purified osteoclasts that were generated in vitro from murine co-culture of marrow precursors with stromal support cells and have found evidence of activation of the MEK/ERK and AKT/NFκB survival pathways. Many mature marrow-derived osteoclasts survived for at least 48 h in culture whether or not they are maintained with stromal cells. Moreover, supplementing purified osteoclasts with RANKL and/or M-CSF had no impact on their survival pattern. In addition, spleen-derived osteoclasts generated with RANKL and M-CSF treatment exhibited a similar survival pattern. Blocking MEK, AKT, or NFκB activity resulted in apoptosis of many, but not all, of the osteoclasts in purified marrow-derived osteoclasts, marrow-derived osteoclasts co-cultured with stromal cells, and spleen-derived osteoclasts maintained with RANKL and M-CSF. These data support that both the MEK/ERK and AKT/NFκB pathways contribute to osteoclast survival. Since PI3K has been shown to activate either of these pathways, we have examined its role in osteoclast survival. PI3K inhibition caused apoptosis of nearly all osteoclasts in purified and co-cultured marrow-derived osteoclasts and spleen-derived osteoclasts maintained with RANKL and M-CSF. Interestingly, in marrow-derived co-cultures, the apoptotic response was restricted to osteoclasts as there was no evidence of stromal support cell apoptosis. PI3K inhibition also blocked MEK1/2, ERK1/2, and AKT phosphorylation and NFκB activation in purified osteoclasts. Simultaneous blockage of both AKT and MEK1/2 caused rapid apoptosis of nearly all osteoclasts, mimicking the response to PI3K inhibition. These data reveal that PI3K coordinately activates two distinct survival pathways that are both important in osteoclast survival. J. Cell. Biochem. 89: 165–179, 2003.

Key words: osteoclast; apoptosis; PI3K; MEK; ERK; AKT; NFκB

Osteoclasts are the multinucleated hematopoetic cells that are responsible for most, if not all, cellular-based bone destruction in vertebrates. Osteoclast numbers are the main determinant of the rate of bone resorption and it is of great interest to understand how osteoclast numbers are controlled [Suda et al., 1997]. It is becoming increasingly evident that osteoclast disappearance in vivo is the result of apoptosis [Lutton et al., 1996; Roodman, 1999]. Apoptosis is a controlled series of events that results in biochemical and morphological changes including membrane blebbing, cell shrinkage, DNA fragmentation, chromatin condensation, and formation of apoptotic bodies. The mechanisms by which mature osteoclasts survive remains unresolved, but conflicting studies have implicated either the PI3K/AKT/NFκB or the MEK/ERK pathways in osteoclast survival [Ozaki et al., 1997; Lacey et al., 2000; Miyazaki et al., 2000; Lee et al., 2001, 2002a,b; Lee and McCubrey, 2002].

The MEK/ERK pathway has been implicated in both survival and apoptosis induction, depending on the signal and the cell system under study [Ishikawa and Kitamura, 1999; Moreno-Manzano et al., 1999; Iryo et al., 2000; Wang et al., 2000; Mitsui et al., 2001]. Miyazaki et al. [2000] examined the roles of MEK in osteoclast survival. This study documented that

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*Correspondence to: Merry Jo Oursler, PhD, University of Minnesota, 211 Life Science, 10 University Drive, Duluth, MN 55812. E-mail: moursler@d.umn.edu

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expression of constitutively active MEK caused enhanced osteoclast survival. In support of a role for this pathway in osteoclast survival, others have demonstrated that ERK activation is important in IL-1 and TNF-α-mediated osteoclast survival [Lee et al., 2001, 2002b].

The AKT/NFκB pathway is important in promoting survival of a spectrum of cell types [Romashkova and Makarov, 1999; Jones et al., 2000; Lee et al., 2000; Madge and Pober, 2000; Madrid et al., 2000; Reddy et al., 2000]. The role of AKT in preventing apoptosis following growth factor withdrawal is well documented, but there is also evidence implicating it in survival in a number of other systems. These include IGF-I, EGF, oxygen stress, TNF-α, IL-1, and PDGF-BB responses in various cells such as osteoblasts, endothelial cells, and epithelial cells [Ahmad et al., 1999; Ushio-Fukai et al., 1999; Madge and Pober, 2000; Wang et al., 2000; Chaudhary and Hruska, 2001; Hong et al., 2001]. There are conflicting reports of the role of NFκB in osteoclast survival. Ozaki et al. [1997] have shown that chemical inhibition of NFκB stimulated osteoclast apoptosis and inhibited resorption, suggesting that NFκB activation regulation is a key component in controlling osteoclast apoptosis. In contrast, Miyazaki et al. [2000] used expression of a dominant interfering form of IKK to inhibit NFκB activation and found that this had no impact on osteoclast survival. Thus, the role of NFκB in the regulation of osteoclast survival and apoptosis is unresolved.

PI3K is a lipid kinase that activates a variety of signaling pathways including the MEK/ERK as well as the AKT/NFκB pathways [Klippel et al., 1997; Scheid and Duronio, 1998; Kelley et al., 1999; Burow et al., 2000; Kuwahara et al., 2000; Lederer et al., 2000; Bisotto and Fixman, 2001; Mograbi et al., 2001; Nosaka et al., 2001; Agazie et al., 2002]. Some studies have documented that PI3K is involved in activation of the MEK/ERK pathway while others have demonstrated that ligand concentration determines whether PI3K impacts this pathway [King et al., 1997; Wennstrom and Downward, 1999; McCubrey et al., 2001; Sheng et al., 2001; von Gise et al., 2001]. There have also been instances in which cross-talk between these pathways have been reported in which the PI3K target AKT directly phosphorylates and inactivates Raf-1 to repress the MEK/ERK pathway [Rommel et al., 1999; Zimmermann and Moelling, 1999; Reusch et al., 2001; Moelling et al., 2002]. Since studies have documented that NFκB and MEK may be involved in modulating osteoclast survival by blocking apoptosis, we have investigated the relationship of these pathways in promoting osteoclast survival.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all chemicals were from Sigma Chemical Co., St. Louis, MO.

Osteoclast Culture and Purification

Mouse marrow and spleen containing osteoclast precursors were obtained from female BalB/c mice (Taconic, Germantown, NY). Four- to six-week-old mice were sacrificed and long bones of the hind limbs and spleen were aseptically removed. The distal ends of bones were clipped and the marrow was flushed out by injecting sterile Mosconas buffer (8% NaCl, 0.2% KCl, 0.06% $NaH_2PO_4 + H_2O$, 2% glucose, 0.02% bicarbonate) into the marrow cavity with a 27-gauge needle. Marrow cells were counted and stored at 2.4×10^6 cells/tube in liquid nitrogen until used. Freezing media consisted of 12% dimethylsulfoxide (DMSO) in fetal bovine serum (FBS) as has been previously reported [Wesolowski et al., 1995]. To generate marrow-derived osteoclasts, precursors were cultured with ST2 stromal cells (Riken Cell Bank, Tsukuba, Japan) during differentiation as follows: apoptosis assessment: ST2 cells were plated in alpha-modified minimal essential media (αMEM, Gibco BRL, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT) and antibiotic/antimycotic in 24-well plates on glass coverslips at a density of 1.6×10^5 cells/well. Biochemical assessments (Western blotting and electrophoretic mobility shift assay (EMSA) analysis): ST2 cells were plated in 6-well plates at a density of 6.4×10^5 cells/well. After 24 h of culture, marrow precursors were plated as follows: for 24-well plates, marrow were added at 1.18×10^5 cells/well, and for 6-well plates, marrow cells were added at 4.75×10^5 cells/well. Precursors were added to the stromal cells using MEM, 10% FBS, 1% non-essential amino acids, and 1% penicillin/streptomycin (base medium). Base medium was supplemented with 7×10^{-3} M ascorbic acid (Gibco BRL, Rockville, MD), 1×10^{-7} M dexamethasone, and 1×10^{-5} M vitamin D_3 (BioMol,

Plymouth Meeting, PA) immediately before use [Takahashi et al., 1988; Udagawa et al., 1990]. Cells were fed every 3 days until 13 days of culture. Osteoclast-like cells are purified by treatment for 15 min at 37°C with 0.2 mg/ml collagenase (Worthington Biochemicals, Lakewood, NJ) in Ham's F12 media (Gibco BRL) followed by 30 min at 37°C with 0.2 mg/ml dispase (Boehringer Mannheim, Framingham, MA) in Ham's F12 media to remove support cells. For generating spleen-derived osteoclasts, spleen cells were plated at 4×10^6 spleen cells per well in a 24-well plate as published papers have detailed for this model system [Sells Galvin et al., 1999]. Base medium was supplemented with 7×10^{-3} M ascorbic acid prior to plating the cells and cultures were supplemented with 30 ng/ml of RANKL (Calbiochem, La Jolla, CA) and 25 ng/ml of macrophage colony stimulating factor (M-CSF) (R&D, Minneapolis, MN). For both marrow- and spleen-derived cultures, media was changed every 3 days and cells were either fixed in 1% paraformaldehyde in phosphate buffered saline to terminate culture or treated as detailed below for individual experiments.

Western Blotting

Osteoclasts were purified as above and either harvested immediately for Western blotting or cultured in MEM for the indicated time period, rinsed with Mosconas buffer, and harvested for Western blotting. Harvesting was accomplished by scraping into SDS sample buffer lacking ß-mercaptoethanol and sodium dodecyl sulfate. To insure that equal cell protein was analyzed, equal (40 μg) cell protein (as determine using BioRad's Protein Quantitation in Detergent Analysis kit) was loaded in each lane. Following protein quantitation, ß-mercaptoethanol and sodium dodecyl sulfate were added to the samples. Parallel Western blotting was carried out as directed in the product literature using antibodies directed against phospho- or total ERK1/2, MEK1/2, or AKT at a 1: 2,000 dilution and secondary antibodies at a 1:10,000 dilution with chemiluminescent detection using the Pierce (Rockford, IL). All antibodies were from Cell Signaling (Beverly, MA).

Apoptosis Detection

Marrow-derived osteoclasts that were either maintained with stromal cells or purified as above were cultured in base media for the indicated time period, fixed with 1% paraformaldehyde and stained. Staining for two apoptotic measures was as given below.

Chromatin condensation. Fixed osteoclasts were stained for 60 min with Hoechst 33258 diluted to 5 μg/ml in phosphate buffered saline with 0.01% Tween 20. The cells were then TRAP stained using a kit from Sigma Chemical Co.

DNA fragmentation. Terminal deoxynucleotide transferase (TdT)-mediated labeling of fragmented DNA with fluorochrome conjugated nucleotides was carried out using the FragEL detection kit, following manufacturer's instructions (Calbiochem). After labeling, cells were examined with fluorescent microscopy, and cells displaying strongly labeled/condensed nuclei were scored as apoptotic.

Pattern of Osteoclast Survival

To examine the timing of osteoclast apoptosis, mature marrow-derived osteoclasts were either maintained with stromal cells or purified as above, and culture conditions were continued as indicated in the figure legend. Spleen-derived osteoclasts were maintained with RANKL and M-CSF throughout culture and treatments. Cells were fixed and then Hoechst and TRAP stained as above.

Inhibition of Signaling Pathway Components

Osteoclasts were generated and then either pretreated for 45 min or purified in the presence of either vehicle (DMSO), the PI3K inhibitors LY294002 (50 μM) or wortmannin (10 μM), the MEK 1 inhibitor PD98059 (50 μM), the MEK1/2 inhibitor U0126 (10 μM), an AKT inhibitor, a phosphatidylinositol ether analog (Calbiochem, San Diego, CA) (5 μM), or the NFκB activation inhibitor pyrrolidine dithiocarbamate (PDTC) (0.2 mM). Osteoclasts were either fixed (time zero) or cultured for 90 min in the continued presence of either vehicle or the indicated inhibitor, and assessed for apoptosis using chromatin condensation as above or analyzed by Western blotting (MEK inhibition and antiphospho ERK Western blot) or EMSA (NFκB inhibition and NFκB activation analysis) as detailed below.

EMSA

Purified osteoclasts were cultured for 0, 5, 10, or 15 min and harvested on ice. Cell pellets were scraped with hypotonic buffer (10 mM Tris-HCl,

pH 7.4, 10 mM NaCl, 30 mM $MgCl_2$, 0.02% sodium azide, and 1 mM each of phenylmethanesulfonyl fluoride, pepstatin, aprotinin, and leupeptin). After 10 min on ice, 0.05% non-idet P-40 was added and nuclei pelleted by centrifugation at 2,500*g* for 5 min. Pellets were washed in the above buffer and resuspended in 20 mM HEPES, pH 7.4, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.01% sodium azide with the above proteinase inhibitors. Nuclear proteins were recovered from the supernatant following centrifugation at 12,000*g* for 10 min. A double stranded oligonucleotide containing the site of interest was end labeled with T4 polynucleotide kinase using γ^{32}P[ATP]. The NFκB target sequence used was: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. The binding assay was performed by incubation of 5 μg of nuclear proteins with 10,000 dpm of the labeled oligonucleotide and 0.5 μg of oligo (dI-dT) in 8.5 mM HEPES, pH 7, 104 mM NaCl, 0.2 mM DTT, 8.5% glycerol for 20 min at 25°C. Treatment was followed by polyacrylamide gel electrophoresis in a 7% non-denaturing gel. Gels were dried and examined by autoradiography. Parallel EMSA of the 15-min nuclear extract in the presence of antibodies (2 μg; Santa Cruz Biotechnology, Santa Cruz, CA) directed against the p65 subunit (p65) was also examined.

Statistical Analysis

The results represent the mean ± SEM of three replicates from one experiment. Each experiment was carried out a minimum of three times and the results shown are representative of all results obtained. The effect of treatment was compared with control values by one-way analysis of variance (ANOVA); significant treatment effects were further evaluated by the Fisher's least significant difference method of multiple comparisons in a ANOVA. Tests were carried out using Apple software, obtained from Statview II, Abacus Concepts, Inc., Cupertino, CA.

RESULTS

Examination of Active Signaling Pathways in Purified Osteoclasts

As an initial study to characterize these cells, osteoclasts were generated from co-culture of marrow and ST2 support cells and purified by enzymatic removal of stromal cells. Cells were immediately harvested and analyzed by

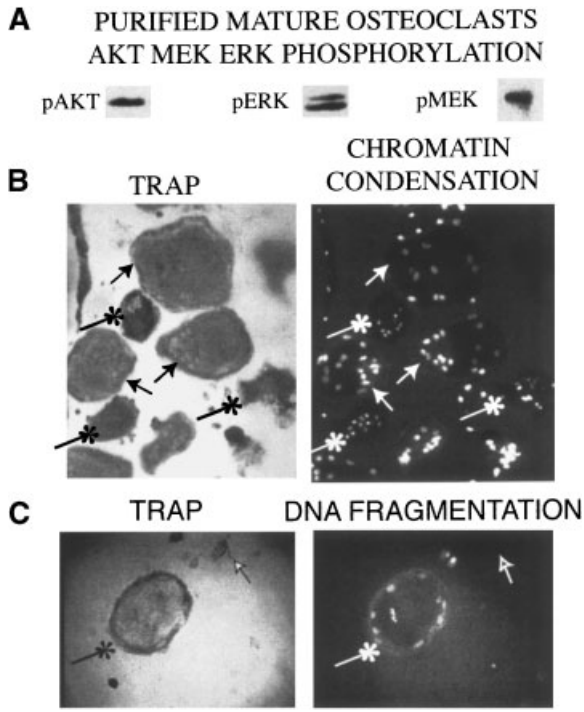

Fig. 1. Characterization of purified osteoclasts. **A**: Mature marrow-derived osteoclasts were purified and immediately harvested. Forty micrograms of protein were analyzed by Western blotting for phospho-AKT (pAKT), phospho-MEK (pMEK), phospho-ERK1/2 (pERK1/2). Evaluation of osteoclast apoptosis. **B**, **C**: Purified marrow-derived osteoclasts were cultured for 90 min, fixed, and stained for TRAP and apoptosis by detecting either chromatin condensation by Hoechst staining (B) or DNA fragmentation staining (C). Arrows point to selected non-apoptotic osteoclasts. The stars indicate selected apoptotic osteoclasts.

Western blotting using phospho-specific antibodies to pAKT, pMEK, and pERK1/2 (Fig. 1A). We observed that there was phosphorylation of AKT, MEK, and ERK1/2 in these cells. By 90 min of culture, there was no detectable phosphorylation of these proteins (data not shown). These data documented that mature osteoclasts showed evidence of transiently activated AKT, MEK, and ERK1/2, intracellular proteins that are reported to promote osteoclast survival. We, therefore, initiated studies to examine whether activation of these proteins in mature osteoclasts had an impact on their survival, the focus of this manuscript. For our first studies, we examined purified osteoclast survival following 90 min of culture using two measures for osteoclast apoptosis, chromatin condensation and DNA fragmentation (Fig. 1B,C). The condensed nuclei (Fig. 1B) and DNA fragmentation staining (Fig. 1C) distinguish apoptotic osteoclasts. Stars point to selected apoptotic osteoclasts

based on Hoechst staining (Fig. 1B) or DNA fragmentation detection (Fig. 1C). The arrows point to selected surviving osteoclasts.

Pattern of Osteoclast Survival in Culture

Having observed that many mature osteoclasts contain active pro-survival intracellular proteins and survived following stromal cell removal, we examined how long osteoclasts could maintain themselves in vitro with and without the presence of stromal cells using TRAP and Hoechst staining. For these studies, we examined mouse osteoclasts from two sources for their survival patterns in vitro: marrow-derived osteoclasts generated in co-culture as above and spleen-derived osteoclasts differentiated in vitro with RANKL and M-CSF (Fig. 2). Once mature, marrow cells co-cultured with stromal cells were either purified as above (Fig. 2A) or maintained in co-culture (Fig. 2B). As documented in the purified osteoclasts graph there was rapid apoptosis of approximately 25% of the osteoclasts present during 90 min of further culture. With longer culture, a significant proportion of surviving osteoclasts continued to show no indication of apoptosis. We have examined cultures for detached osteoclasts by cytospinning culture supernatants and have found a few apoptotic bodies. Due to the inability to determine cell numbers from these, we are restricting our analysis to counting apoptotic intact osteoclasts. However, the total number of osteoclasts did not appreciably decrease over time, suggesting that there were few cells released from the

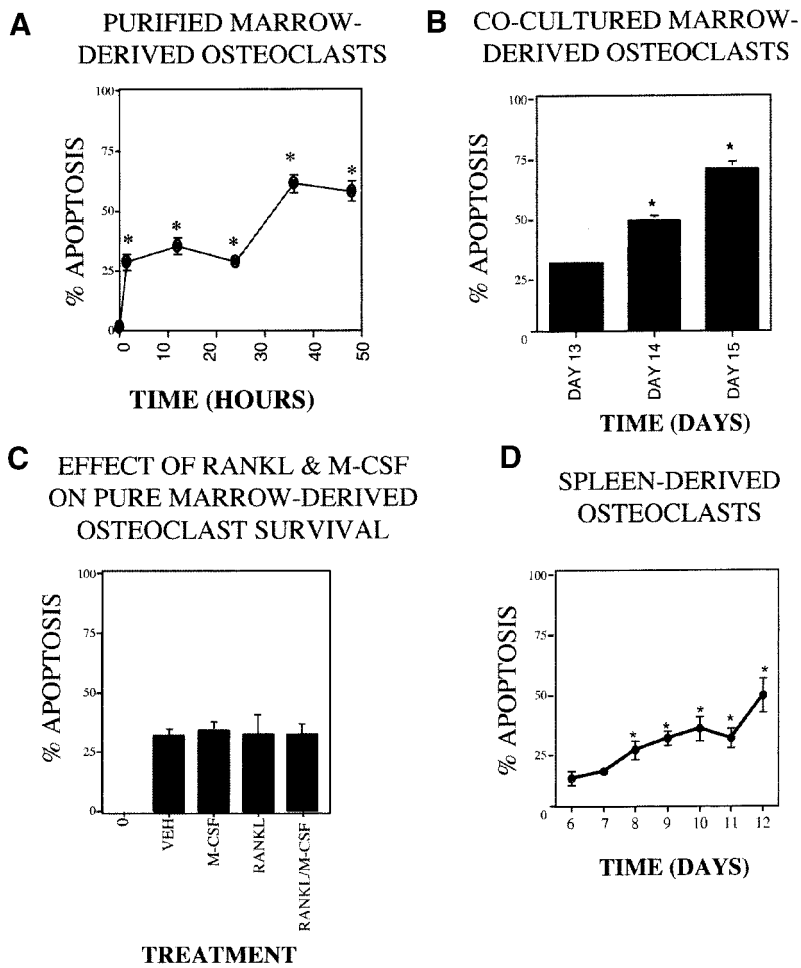

Fig. 2. The pattern of osteoclast apoptosis. Marrow-derived osteoclasts were either purified and cultured for the indicated time (**A**), fixed at the indicated time as co-cultures (**B**), or purified and cultured with 10 ng/ml RANKL and/or 25 ng/ml M-CSF as indicated (**C**). Spleen-derived osteoclasts were maintained with RANKL and M-CSF and analyzed on the indicated day of culture (**D**). The cells were stained for TRAP and chromatin condensation. Apoptotic osteoclasts were identified as TRAP stained cells having highly condensed nuclei. The percentage of apoptotic osteoclasts is shown (mean ± SEM of three replicates). $^*P < 0.05$ compared to earliest time point.

coverslip. These studies raised the possibility that stromal cell removal may be a trigger for osteoclast apoptosis. Since osteoclasts do not exist in vivo in the absence of stromal cells, we have examined mature osteoclast cultures maintained with stromal cells for apoptotic osteoclasts over a time period similar to the above studies (Fig. 2B). On culture day 13, approximately 25% of the osteoclasts were apoptotic. Over the following 2 days, the proportion of apoptotic osteoclasts further increased on each successive day during co-culture with support cells. The discrepancy between the purified (Fig. 2A) and co-culture day 13 time point (Fig. 2B) is likely due to removal of apoptotic osteoclasts from the coverslip when the stromal cells were enzymatically removed. We have also examined the influence of replacing RANKL and/or M-CSF to the purified osteoclasts (Fig. 2C) and observed that replacing RANKL or M-CSF or both for 90 min of culture had no impact on apoptosis. To examine mouse osteoclasts from a precursor population that has few, if any, stromal cells, we used spleen cells cultured without stromal cells as another model systems (Fig. 2D). Spleen-derived osteoclasts were fixed after the indicated time in culture, stained, and evaluated as outlined above. As documented in Figure 2D, there were some apoptotic osteoclasts appearing by the first day of analysis, day 6. There was a significant elevation in apoptosis above this level beginning at day 8 and lasting throughout the analysis time. As with the marrow-derived osteoclasts above, there was significant numbers of surviving osteoclasts in these cultures as well.

Examination of Osteoclast Survival Mechanisms

Our time course studies document that significant numbers of osteoclasts were surviving in culture whether or not stromal cells, M-CSF or RANKL were present and we have, therefore, pursued the mechanisms by which they survive. We have focused on the MEK/ERK and AKT/NFκB pathways as they are two signaling pathways that have been well-documented to promote survival in a number of cell systems and our data above support that they are activated in purified osteoclasts.

Role of the MEK/ERK pathway in osteoclast survival. Initial studies of whether the MEK/ERK pathway might be involved in osteoclast survival focused on examining the cultures for evidence of activation of MEK and ERK (Fig. 3). Due to the need to utilize the model system with the purest osteoclast population, Western blot analyses were carried out on purified marrow-derived osteoclasts. We are cognizant that this is an artificial system in that osteoclasts do not exist in vivo in the absence of stromal cells, so all inhibition analyses are being done with marrow-derived osteoclasts with and without stromal cells and spleen-derived osteoclasts maintained with M-CSF and RANKL. As seen by Western blot analysis (Fig. 3A), within 5 min of culture, there was increased phosphorylation of MEK1/2 and ERK1/2. The most likely explanation for this observation is that the addition of serum (following its absence as the result of the 45 min of culture during the treatment period when stromal cells were removed) caused this response. At time zero, there was some evidence of MEK and ERK phosphorylation, although to a lesser extent than the culture response. To verify that inhibiting MEK1/2 blocked ERK activation in our cultures we initially determined whether 10 μM of the MEK1/2 inhibitor U0126 blocked ERK phosphorylation. As shown in Figure 3B, this dose effectively blocked phosphorylation of ERK1/2. These data documented that we were effectively blocking MEK-mediated ERK activation, so we examined the impact of blocking MEK1/2- and Raf (which can activate MEK) on osteoclast survival. As revealed in Figure 3C,D, inhibition of either Raf or MEK1/2 resulted in apoptosis of over half of the osteoclasts within 90 min of treatment independent of whether the osteoclasts were purified or maintained with stromal cells, supporting an involvement of the Raf/MEK1/2-ERK1/2 pathway in marrow-derived osteoclast survival. Following purification in Figure 3C, there was a significant level of apoptosis in the cultures (0 time). This is likely due to the inclusion of the inhibitors during the 45-min purification process. We next examine spleen-derived osteoclasts and observed that inhibition of either Raf or MEK1/2 for 2 h significantly increased osteoclast apoptosis in these cultures (Fig. 3E), supporting a role for the Raf/MEK/ERK pathway in spleen-derived osteoclast survival.

Role of the AKT/NFκB pathway in osteoclast survival. Since we detected phosphorylated AKT in freshly isolated osteoclasts and the AKT/NFκB pathway has also been implicated in promoting survival of a spectrum

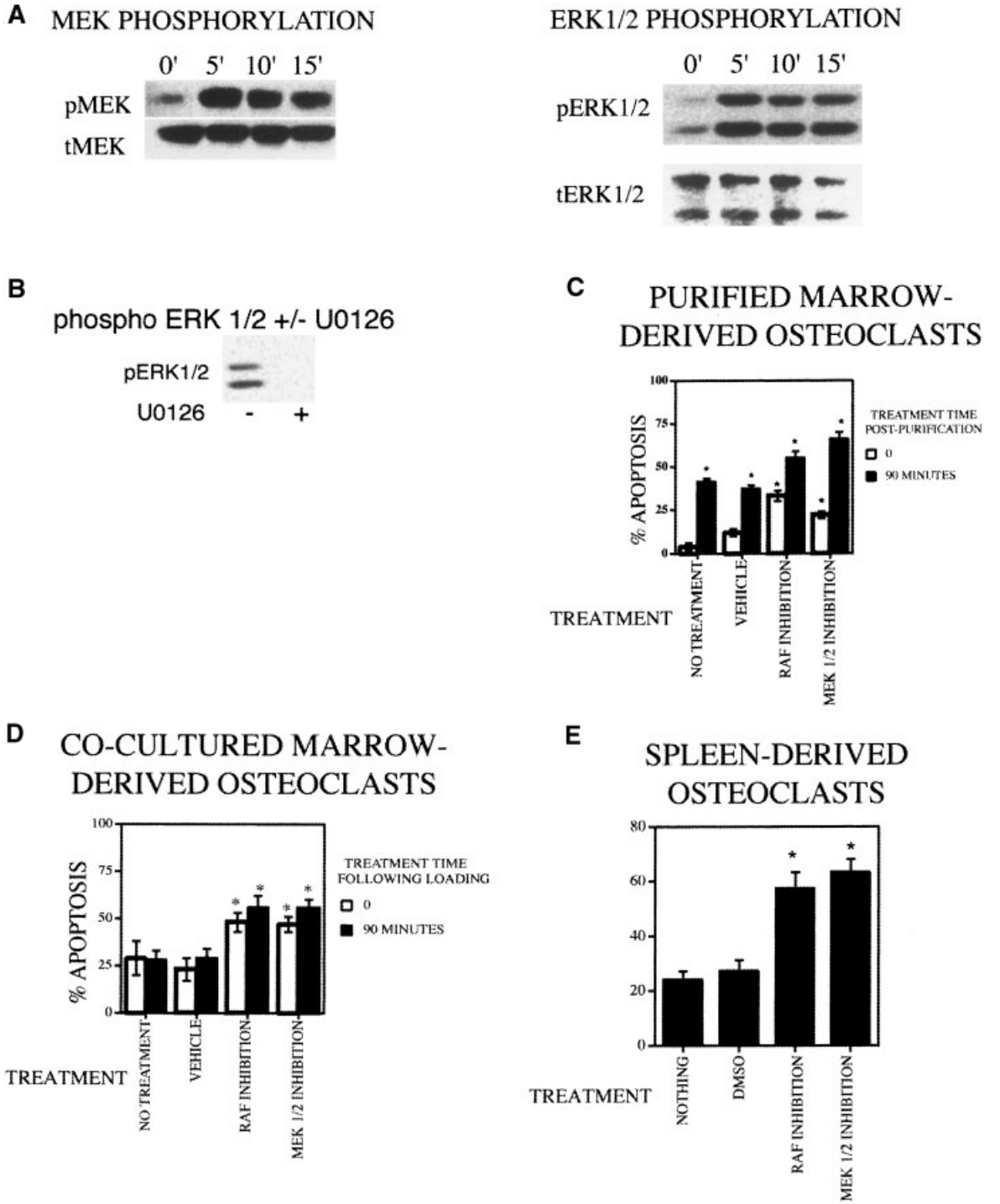

Fig. 3. The MEK/ERK pathway is involved in osteoclast survival. Activation of MEK1/2 and ERK1/2 during culture. **A**: Purified marrow-derived osteoclasts were cultured for 0–15 min as indicated and harvested for Western blotting for pMEK or total MEK (tMEK), pERK or total ERK (tERK) as described. The effects of inhibition. **B**: Marrow-derived osteoclasts were treated with the MEK1/2 inhibitor U0126 during purification and subsequent 15 min of culture. Cells were harvested for Western blotting for pERK as described. **C**, **D**: Marrow-derived osteoclasts were purified in the presence of vehicle, a Raf inhibitor, or the MEK1/2 inhibitor (C) or pre-treated with vehicle or the inhibitors for co-culture analysis (D). Cells were either fixed (0 culture time) or cultured in the presence of the same treatment for 90 min. **E**: Spleen-derived osteoclasts were cultured for 2.5 h with vehicle or the indicated inhibitor. After fixation, cells were stained for TRAP and chromatin condensation. Apoptotic osteoclasts were identified as above. The percentage of apoptotic osteoclasts is shown (mean ± SEM of three replicates). $^*P < 0.05$: for 0 time, compared to vehicle and for 90 min cultures, compared to both vehicle and 0 time of same treatment.

of cell types, we explored this pathway for whether it may have a role in osteoclast survival (Fig. 4). As above, we utilized purified osteoclasts from co-cultures of marrow precursors with stromal cells for Western blot analyses and examined the impact of blocking pathway components using multiple model systems. We first examined the impact of culture on osteoclast AKT phosphorylation (Fig. 4A). Within 5 min of culture, there was a striking increase in AKT phosphorylation that persisted through 15 min of culture. Note that the time zero sample again showed evidence of AKT phosphorylation. Following the indication of AKT activation, we examined the AKT target NFκB since it influences survival in many cell types [Ozes et al., 1999; Xie et al., 2000; Madrid et al., 2001]. We initially sought to determine if NFκB had a potential role in osteoclast survival by assaying for changes in NFκB activation during culture (Fig. 4B). EMSA analysis of purified osteoclast nuclear extracts revealed that there was evidence of activation at time zero and a rapid elevation in NFκB activation and nuclear localization when cultured further. To verify that we were detecting NFκB, we performed parallel EMSA of the 15-min nuclear extract in the presence of antibodies directed against the p65 subunit (Fig. 4C). Based on the appearance of a higher molecular weight complex concomitant with a decrease in the intensity of the band at the shifted location, we have documented that this band in this EMSA is the complex of NFκB and labeled oligo. Conflicting reports have suggested that NFκB may be involved in osteoclast activation but perhaps not in survival [Ozaki et al., 1997; Miyazaki et al., 2000]. We have, therefore, employed chemical blocking of both AKT and NFκB to study the role of the AKT/NFκB pathway in osteoclast survival in culture. Initially, we examined 0.2 mM of the NFκB activation inhibitor PDTC to determine that it was effective at blocking NFκB activation and found that we were using an effective blocking dose (Fig. 4D). We then examined the impact of blocking AKT or NFκB on osteoclast survival (Fig. 4E–G). Within 90 min of culture with either inhibitor, roughly half of the osteoclasts were apoptotic in all model systems examined, documenting that AKT and NFκB participate in osteoclast survival independent of whether osteoclasts are cultured as purified cells (Fig. 4E), maintained with support cells (Fig. 4F), or maintained with RANKL and M-CSF (Fig. 4G). As above, the apoptosis that was measured at treatment time 0 in purified osteoclasts (Fig. 4E) is likely due to the 45-min pretreatment during purification. AKT promotes survival by phosphorylating a number of targets including caspase 9, Bad, GSK-3, and FRAP/mTOR, which targets p70 S6 kinase [Datta et al., 1997; Fujita et al., 1999; Gingras et al., 1999; Khaleghpour et al., 1999; Raught and Gingras, 1999; Beitner-Johnson et al., 2001; Gingras et al., 2001]. Our data do not support that these targets are activated in osteoclasts, so these have not been pursued further (data not shown). We conclude from these data that AKT and NFκB are involved in promoting osteoclast survival.

Role of PI3K in osteoclast survival. Since PI3K is a multifunctional second messenger that has been implicated in activating a number of different survival signaling pathways, we examined whether blocking PI3K activity influenced osteoclast survival. As with the preceding blocking studies, we examined pathway blocking using marrow-derived osteoclasts with and without purification as well as spleen-derived osteoclasts. Figure 5A is a photograph of a co-culture of marrow-derived osteoclasts cultured with stromal support cells that has been incubated with 10 μM wortmannin for 2.5 h. In the picture, the TRAP staining photograph taken with visible light was over-layered with the fluorescent Hoechst stained same field of view. The larger nuclei (selected ones are indicated with arrows) that are surrounding and over-laying the osteoclasts are the nuclei of the TRAP-negative mononuclear stromal cells that overlay and surround the purple TRAP-positive multinucleated osteoclasts (indicated with the stars). Interestingly, the 2.5-h treatment that resulted in apoptosis of nearly all of the osteoclasts co-cultured with stromal cells, there was no detectable apoptosis of the stromal cells. We quantitated the apoptotic response of purified osteoclasts, osteoclasts maintained with stromal cells, and spleen-derived osteoclast. Inhibition of PI3K activity with either LY294002 or wortmannin resulted in apoptosis of nearly all of the osteoclasts in each of the model systems examined (Fig. 5B–D).

Role of PI3K in regulating the MEK/ERK and AKT/NFκB pathways. To determine whether PI3K was involved in activation of either the MEK/ERK or AKT/NFκB pathway in surviving osteoclasts, we examined the impact of PI3K inhibition on both of these

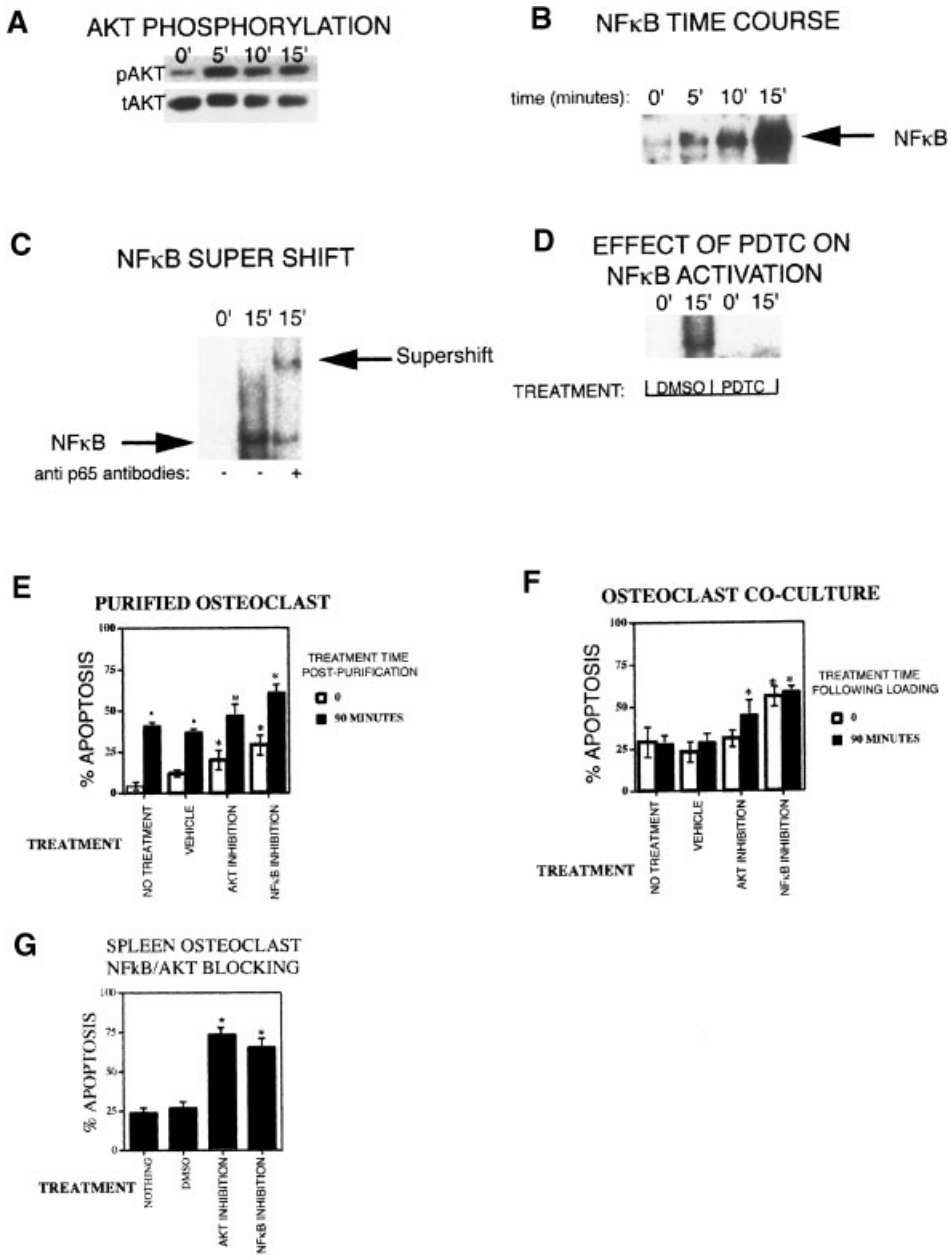

Fig. 4. The AKT/NFκB pathway is involved in osteoclast survival. Activation of AKT/NFκB during culture. **A**–**C**: Purified marrow-derived osteoclasts were cultured for 0–15 min as indicated and harvested for Western blotting for pAKT or total AKT (tAKT) as described (A). Purified marrow-derived osteoclasts were cultured for 0–15 min as indicated and harvested for nuclear extraction and electrophoretic mobility shift assay (EMSA) analysis as described (B). As detailed in Experimental Procedures, 15-min extracts were assessed for the presence of p65 subunit by the addition of anti-p65 antibodies prior to gel analysis (+ lane) (C). The effects of inhibition. **D**–**G**: Marrow-derived osteoclasts were treated with either vehicle or 0.2 mM of the NFκB activation inhibitor pyrrolidine dithiocarbamate (PDTC) during purification and subsequent 15 min of culture. Cells were harvested after purification or culture for NFκB activation as described (D). Marrow-derived osteoclasts were either purified in the presence of vehicle, AKT or NFκB activation inhibitor (E) or pre-treated with the inhibitors for co-culture analysis (F). Spleen-derived osteoclasts were cultured for 2.5 h with vehicle or the indicated inhibitor (G). Cells were either fixed (0 culture time) or cultured in the presence of the same treatment for 90 min. After fixation, cells were stained for TRAP and chromatin condensation. Apoptotic osteoclasts were identified as above. The percentage of apoptotic osteoclasts is shown (mean ± SEM of three replicates). $^*P < 0.05$: for 0 time, compared to vehicle and for 90 min cultures, compared to both vehicle and 0 time of same treatment.

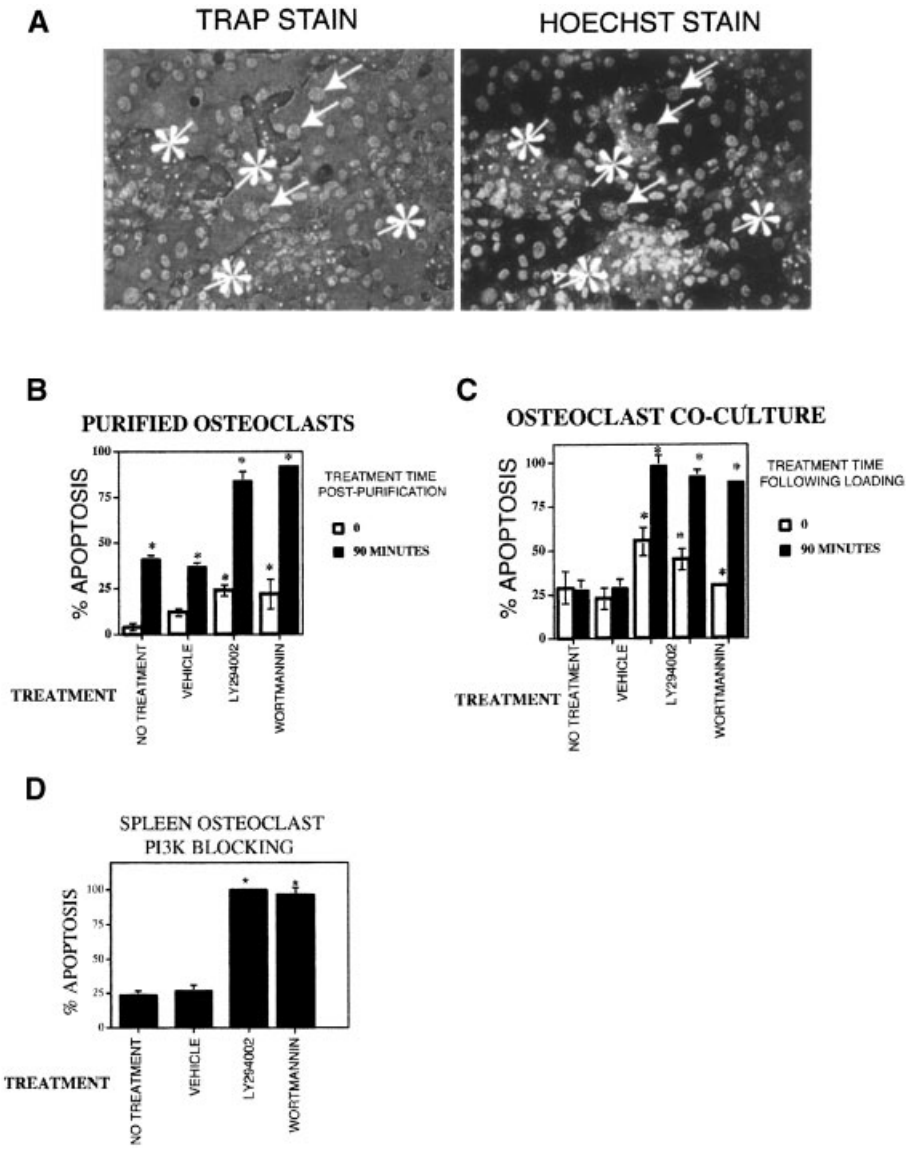

Fig. 5. PI3K mediates osteoclast survival. Examination of the impact of PI3K inhibition of co-cultures. **A**: Marrow-derived osteoclasts maintained with ST2 cells were treated for 2.5 h with wortmannin, fixed and stained for TRAP and chromatin condensation. Arrows point to selected non-apoptotic ST2 cells in the co-cultured cells. The stars indicate selected apoptotic osteoclasts in both pure and co-cultures. The effects of inhibition. **B**–**D**: Marrow-derived osteoclasts were either purified in the presence of vehicle or one of two PI3K inhibitors LY294002 or wortmannin (B) or pre-treated with the inhibitors for co-culture analysis (C). Cells were either fixed (0 culture time) or cultured in the presence of the same treatment for 90 min. Spleen-derived osteoclasts were cultured for 2.5 h with vehicle or the indicated inhibitor (D). After fixation, cells were stained for TRAP and chromatin condensation. Apoptotic osteoclasts were identified as above. The percentage of apoptotic osteoclasts is shown (mean ± SEM of three replicates). $*P < 0.05$: at 0 time, compared to vehicle and at 90 min cultures, compared to both vehicle and 0 time of same treatment.

pathways. As documented in Figure 6A, both PI3K inhibitors blocked phosphorylation of AKT. Since the PI3K inhibitors blocked basal osteoclast survival and AKT phosphorylation, we examined if blocking PI3K also blocked osteoclast NFκB activation (Fig. 6B). Blocking PI3K effectively blocked osteoclast NFκB activation, verifying that PI3K was initiating a cascade that results in NFκB activation in cultured osteoclasts. In many cell types, PI3K activation results in stimulation of MEK-mediated phosphorylation of ERK1/2, so we examined whether blocking PI3K impacted the rapid phosphorylation of MEK1/2 and ERK1/2 that we detected (Fig. 6C,D). We observed that PI3K inhibition blocked phosphorylation of both

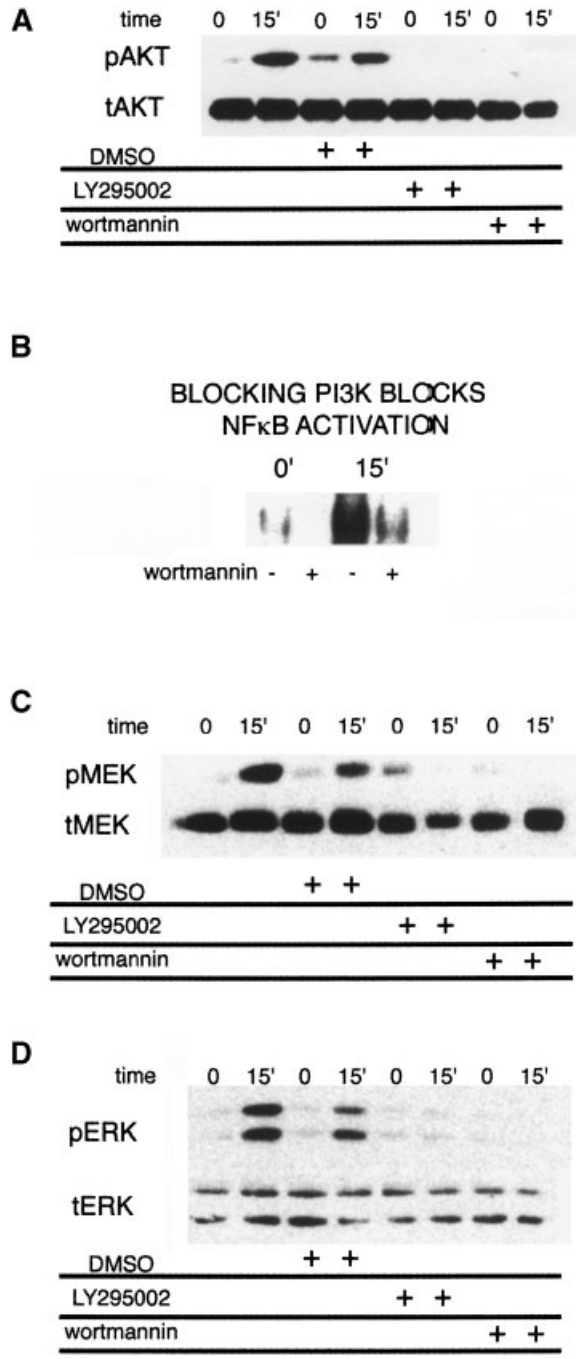

Fig. 6. PI3K coordinately regulates both MEK/ERK and AKT/NFκB pathway activation. The effects of PI3K inhibition on phosphoprotein levels. **A**–**D**: Osteoclasts were either purified in the presence of vehicle or one of two PI3K inhibitors LY294002 or wortmannin and either harvested (0) or cultured for 15 min (15′) with the same treatment. Cells were harvested for Western blotting for pAKT or tAKT (A), pMEK or tMEK (C), or pERK or tERK (D) as described. The effects of PI3K inhibition on NFκB activation. B: Osteoclasts were either purified in the presence of vehicle or wortmannin and either harvested (0) or cultured for 15 min (15′) with the same treatment. Nuclear extracts were assessed for activation and nuclear localization of NFκB by EMSA analysis as described.

MEK and ERK in the osteoclast cultures. We conclude from these data that PI3K coordinately activates both the AKT/NFκB and MEK/ERK pathways to promote osteoclast survival.

Impact of blocking both AKT and MEK1/2 on osteoclast survival. Since individual blocking of AKT or MEK1/2 did not induce apoptosis of all of the osteoclasts, we examined the impact of blocking both of these pathways together (Fig. 7). Simultaneous blockage of both pathways resulted in apoptosis of nearly all osteoclasts in both purified cultures (Fig. 7A), co-cultures with stromal cells (Fig. 7B), or spleen-derived osteoclasts maintained with M-CSF and RANKL (Fig. 7C). This pattern mimics the pattern observed when the PI3K pathway was blocked with either LY294002 or wortmannin.

DISCUSSION

We have examined mature osteoclast survival using a well-documented in vitro differentiation system to generate large numbers of mouse osteoclasts [Udagawa et al., 1990]. Others have reported that, once purified, these osteoclasts rapidly apoptose [Fuller et al., 1993]. We have examined these cultures and found that there was evidence of maintained activation of the AKT/NFκB and MEK/ERK pathways. These observations are in concordance with the studies of Miyazaki et al. [2000] and Xing et al. [2001] documenting activation of osteoclast ERK and AKT, respectively. Further study of the cultured osteoclasts derived from marrow or spleen precursors reveal that, although many mature osteoclasts apoptosed once support cells were removed, a significant number survived up to 48 h following purification. Examination of marrow-derived osteoclast cultures in which stromal cells were not removed revealed that, by day 13 of culture, there were significant numbers of apoptotic osteoclasts and that this number increased with culture time. In the purified day 13 osteoclasts, we were not able to detect these apoptotic cells prior to subsequent culturing. This discrepancy is most likely due to efficient removal of apoptotic osteoclasts during the purification steps on day 13. Further culture of both purified osteoclasts and co-cultures resulted in additional apoptotic osteoclasts, although significant numbers of osteoclasts survived. Moreover, addition of RANKL and/or M-CSF to purified osteoclasts

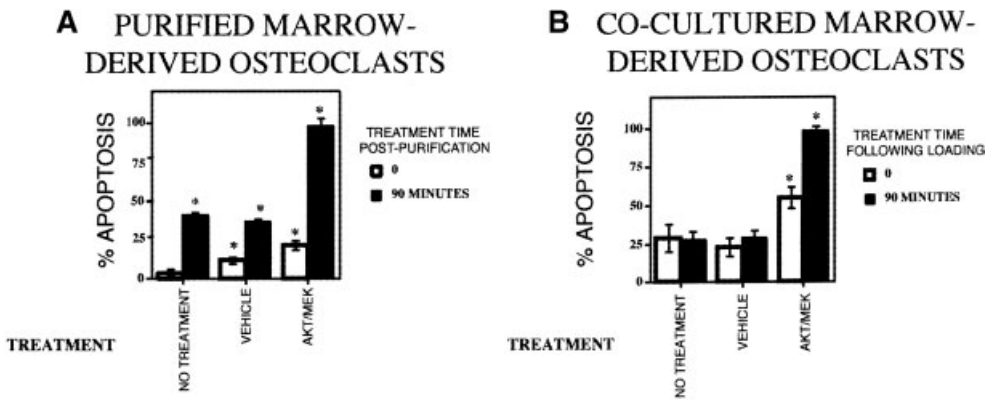

Fig. 7. Inhibition of both AKT and MEK mimic the effects of PI3K on osteoclast survival. Osteoclasts were either purified in the presence of vehicle, or the AKT and the MEK1/2 inhibitor U0126 combined together (AKT/MEK) (**A**) or pre-treated with the same inhibitors for co-culture analysis (**B**). Cells were either fixed (0 culture time) or cultured in the presence of the same treatment for 90 min. After fixation, cells were stained for TRAP and chromatin condensation. Apoptotic osteoclasts were identified as above. The percentage of apoptotic osteoclasts is shown (mean ± SEM of three replicates). $*P < 0.05$: for 0 time, compared to vehicle and for 90 min cultures, compared to both vehicle and 0 time of same treatment.

did not measurably impact the rate of survival. These data support that the removal of the stromal cell-derived RANKL or M-CSF was not the trigger for apoptosis but, rather, that osteoclasts maintain a rate of apoptotic death independent of whether stromal cells were present. Spleen-derived osteoclasts were generated by culture with RANKL and M-CSF. Maintenance of these cultures with RANKL and M-CSF did not prevent apoptosis of some of the osteoclasts. Taken together, these data support that there is a sustained rate of apoptosis of some osteoclasts while others survive in culture. The focus of the remainder of this study was on the mechanisms by which the cultured osteoclasts survived.

Our studies provided evidence of activation of both the MEK/ERK and AKT/NFκB pathways in surviving osteoclasts. Chemical blocking of either of these pathways increased osteoclast apoptosis independent of stromal cells or RANKL and/or M-CSF. Interestingly, significant numbers of osteoclasts survived with separate blockage of each of these pathways whereas blocking PI3K resulted in apoptosis of nearly all osteoclasts. Combined blocking of the AKT/NFκB and MEK/ERK pathways mimicked the PI3K blocking response, suggesting that co-repression of these pathways induced apoptosis in more osteoclasts than separate blocking of each pathway osteoclast apoptosis. To better explore the integration of these signaling pathways, we examined the impact of blocking PI3K on phosphorylation of MEK, ERK, and AKT as well as NFκB activation and documented that inhibition effectively blocked activation of these downstream kinases. These data strongly support that PI3K coordinately activates two survival pathways that have been shown in many survival signaling systems to act independently [Xue et al., 2000; Mograbi et al., 2001; Tsakiridis et al., 2001; Agazie et al., 2002]. Recent studies have shown that cross-talk between the AKT and MEK/ERK pathways in muscle cells and breast cancer cells involves AKT-mediated inactivation of the MEK/ERK pathway. Direct phosphorylation of Raf-1 by AKT repressed the MEK pathway, switching MCF-7 breast cancer cell line response from proliferation cell cycle arrest [Zimmermann and Moelling, 1999]. Moreover, MCF-7 cells are regulated in a ligand- and concentration-dependent manner to either stimulate proliferation in response to insulin-like growth factor I, again by selective PI3K-mediated repression of the MEK/ERK pathway [Moelling et al., 2002]. In myoblasts, there was differentiation stage-specific inhibition of the MEK/ERK pathway in response to IGF-I-mediated AKT activation [Rommel et al., 1999]. In vascular smooth muscle cells, platelet-derived growth factor stimulation the PI3K/AKT pathway to promote differentiation required PI3K-mediated repression of the Raf/MEK/ERK pathway [Reusch et al., 2001]. Thus, to our knowledge, these are the first studies to report that PI3K activates both the AKT/NFκB and the MEK/ERK within the same time frame to participate coordinately in survival.

Although many cytokines and other growth factors impact osteoclast differentiation, there are a more restricted number of external stimuli

that have been documented to influence osteoclast survival. Among these, M-CSF, RANKL, IL-1, and TNF-α have all been shown to promote osteoclast survival. Interestingly, IL-1 and TNF-α both promote osteoclast survival by activation of both the ERK1/2 and the PI3K/AKT pathways. Surprisingly, TNF-α, although activating AKT, does not activate its well-documented target NFκB in osteoclasts [Lee et al., 2001]. Miyazaki et al. [2000] examined a role for NFκB in osteoclast survival using expression of a dominant interfering form of IKK to block NFκB activation. The data suggested that NFκB activation was not necessary for osteoclast survival. These results are in conflict with the results of Ozaki et al. [1997] using chemical inhibition of NFκB and Lacey et al. [2000] showing that RANKL-mediated activation of NFκB is important in osteoclast survival. In the studies reported here, blocking AKT or NFκB effectively blocked survival of a significant number of osteoclasts, although many cells survived this treatment. These data support that at least some osteoclasts require AKT/NFκB activation for survival.

The published literature supports a role for both AKT/NFκB and MEK/ERK in osteoclast survival following inflammatory cytokine stimulation. We report here for the first time that PI3K coordinately activate these pathways to promote osteoclast survival in the absence of inflammatory cytokine treatment. To our knowledge, this is the first report of the role of PI3K in coordinately activating these pathways to support osteoclast survival. Excessive bone loss is a major pathology in several diseases including periodontitis, postmenopausal osteoporosis, glucocorticoid-induced osteoporosis, and metastatic tumor-driven osteolysis. These disparate diseases share a common denominator in the elevation in the numbers of osteoclasts present during bone degradation. Since osteoclast numbers are controlled by impacting the rates of differentiation and elimination by apoptosis, understanding the mechanisms by which osteoclasts survive may be important to future therapeutic designs to limit the number of osteoclasts.

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