INTRODUCTON:

One of the most significant advances in understanding the pathogenesis of bone metastases has been the discovery that osteoclastogenesis occurs at sites of tumor (1), a feature common to both primary bone sarcomas and metastatic breast cancers. However, most therapies have been directed at developing drugs that block bone resorption, such as bisphosphonates. While these therapies have decreased the number of skeletal events, they have had no impact on eliminating the tumor (2) and in humans, osteoclast inhibition does not have the profound effect on tumor burden that was evident in experimental animal models. New therapies are needed to eliminate both osteoclasts and bone cancer cells.

Among the many new experimental approaches, gene therapy has gained particular attention; in particular, the targeting of accessory cells recruited by the tumor as gene delivery vehicles. Recent reports utilizing endothelial precursor cells in lung cancer (3) or neural progenitor cells in gliomas (4) have demonstrated some promise. As a first step toward developing novel therapies to reduce cancer burden in the skeleton, we tested the possibility that osteoclasts could be used as gene delivery vehicles by providing a therapeutic gene at sites of bone tumors in order to direct tumor killing.

In this investigation we describe an osteoclast-mediated, tumor targeted gene therapy, which affects both osteoclasts and cancer cells. We demonstrate effectiveness both in vitro and in vivo. This approach involves using the yeast cytosine deaminase (CD)/5-FC prodrug system, a system in which CD converts the prodrug, 5FC, to the chemotherapeutic drug, 5FU. We hypothesized that 5FC treatment of CD expressing osteoclasts would cause both direct killing of osteoclasts and bystander killing of cancer cells.

METHODS:

In vitro systems — Osteoclasts were cultured from CD transduced RAW 267.4 cells (RAW/CD), CD transduced normal bone marrow (BM/CD) or BM from a transgenic mouse (5) with the CD gene regulated by the TRAP promoter (Tg/TRAP-CD). Constructs contained a fusion gene composed of a truncated human nerve growth factor receptor (NGFR) as a marker and the yeast CD gene (6). For direct osteoclast killing, 5FC [10 to 1000 µM] was added during the first 3 days of culture and osteoclasts were assessed at 6 days using a TRAP solution assay. For bystander killing of cancer cells, fluorescently-labeled sarcoma or breast cancer cells were added to cultured osteoclasts at day 3, 5FC added on days 3 and 5 and fluorescence read on day 7. Killing assays were normalized to cultures without 5FC and repeated at least 3 times. Expression of NGFR or CD was determined by flow cytometry, western analysis and immunocytochemistry (ICC). CD function was determined spectrophotometrically by conversion of 5FC to 5FU.

In vivo experiments — Direct killing of osteoclasts was evaluated after 1 or 4 daily doses of 5FC by histomorphometry and osteoclast generation from BM of 5FC treated mice. Bystander killing was determined by femoral injection of bone cancer cells into Tg/TRAP-CD mice. Six days after implantation of osteolytic tumors mice were treated with 4 days of 5FC and histomorphometric evaluation of medullary tumor and osteoclast number performed. To determine the feasibility of osteoclasts in gene therapy, syngeneic C3H mice were transplanted with Tg/TRAP-CD BM (C3H-BMT). Engraftment was determined by expression of CD by qualitative, real-time RT-PCR and hematopoietic repopulation. Bone cancer was implanted into femora of C3H-BMT mice, treated with 5FC and evaluated for tumor killing.

RESULTS:

In vitro systems — Treatment with 5FC resulted in a dose dependent decrease in osteoclast number in all CD-containing culture systems (p<0.001) but not culture systems containing osteoclasts from normal or non-transduced progenitors [direct killing]. ED₅₀ ranged from 0.6 µM for RAW/CD, 35 µM for BM/CD and 100 µM in Tg/TRAP-CD cultures. Reduction in the number of fluorescent cancer cells was evident in all CD-containing co-culture systems (p<0.001) but not culture systems containing only tumor cells or co-cultures from normal or non-transduced progenitors [bystander killing]. Exposure to 10 µM 5FC resulted in significant reduction in the number of either sarcoma or breast cancer cells (p<0.02). ED₅₀ values were 20 µM for both cell lines using transduced progenitors and 50-80 µM with transgenic progenitors.

In vivo experiments — A single dose of 5FC showed a modest and transient reduction in osteoclast number and size in Tg/TRAP-CD mice. Four daily doses had no effect on osteoclast number and a demonstrated significant (p<0.05) increase in size (219 vs. 154 µm²) compared to untreated mice. In contrast, when BM from treated mice was plated to form osteoclasts in vitro, TRAP enzyme activity was 11% to 19% of untreated BM for up to 7 days after 5FC treatment indicating a severe decrease in osteoclast progenitors. No osteoclasts formed in vitro when BM from mice treated with 4 doses of 5FC was plated.

PCR findings demonstrated a 4-fold increase in endogenous TRAP gene expression and a 2-fold increase CD expression in tumor-bearing, compared to non-injected contralateral (control) femora (p<0.001). 5FC treatment of tumor-bearing animals resulted in reduction in both tumor burden and osteoclast number. Specificaly, untreated femora averaged 89% tumor/necrotic tumor and 11% healthy tissue while 5FC treated femora had 7% tumor and 93% healthy/reparative tissue (p<0.0001). Osteoclast number was 2-fold higher in tumor-bearing femora compared to tumor-bearing/5FC-treated femora (p<0.01), numbers comparable to untreated femora.

Analysis of femora from C3H-BMT mice revealed CD expression equivalent to that seen in donor animals by 8 weeks after transplant. Osteoclasts cultured from C3H-BMT marrow expressed NGFR by ICC and, when exposed to 5FC, demonstrated a dose dependent decrease in osteoclast number in vitro.

DISCUSSION:

Findings from this investigation demonstrate that CD-containing osteoclasts can direct killing of sarcoma and breast cancer cells in vitro. In vivo, Tg/TRAP-CD mice received osteolytic bone sarcoma that after 5FC treatment resulted in elimination of tumor and reduction in osteoclast number. Transplant of Tg/TRAP-CD BM into normal mice demonstrated engraftment, expression of CD, and in vitro killing of osteoclasts.

Development of osteoclast-mediated, tumor-targeted enzyme prodrug therapies may be the logical step to follow the progress made with bisphosphonate treatment. This investigation provides the basis for developing such a treatment for primary or metastatic bone cancers, which would simultaneously kill tumor cells and eliminate osteoclast-mediated bone destruction.

REFERENCES: