Clodronate inhibits angiogenesis in vitro and in vivo

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Abstract. The effects of amino-bisphosphonate clodronate on endothelial cell functions involved in angiogenesis, namely proliferation and morphogenesis on matrigel were tested in vitro, whereas its effects on angiogenesis were studied in vivo. This was performed by using the chick embryo chorioallantoic membrane (CAM) assay. In vitro, clodronate inhibited the endothelial cell proliferation in a dose-dependent fashion, peaking at 30 μM. At the same concentration, clodronate inhibited the fibroblast growth factor-2 (FGF-2)-induced capillary-like tube formation in the morphogenesis assay on matrigel. In vivo, when tested with the CAM assay, clodronate again displayed the capability to inhibit FGF-2-induced angiogenesis. Overall, these results suggest that antiangiogenesis by clodronate can be used to treat a wide spectrum of angiogenesis-dependent diseases, including certain chronic inflammatory diseases and cancer.

Introduction

Bisphosphonates (BP) are analogues of PPI and differ from one another based on their substituted side chain (1). They can be segregated into two distinct pharmacological classes [i.e., nitrogen containing (amino) and non-nitrogenic-containing bisphosphonates] based on their molecular mechanism of action (2).

Some evidence suggests that part of the antitumor activity of bisphosphonates may be attributed to an antiangiogenic effect. Wood et al (3) reported that endothelial cell proliferation and migration in vitro induced by the fibroblast growth factor-2 (FGF-2), the vascular endothelial growth factor (VEGF) and angiogenesis in vivo in the chick embryo chorioallantoic membrane (CAM) assay are inhibited by zoledronic acid. The same investigators demonstrated that in a subcutaneous growth factor implant model in mice, zoledronate treatment strongly inhibited the angiogenic response induced by FGF-2 and VEGF (4).

Fournier et al (5) analyzed the role of bisphosphonates on the inhibition of endothelial cell functions both in vitro and in vivo. Four bisphosphonates were tested in vitro: clodronate, ibandronate, risedronate and zoledronic acid. In vivo studies were performed by quantification of the vascularization in bone biopsy specimens from patients with Paget’s disease before and after clodronate treatment and by analysis of testosterone-induced revascularization of the prostate gland in castrated rats. Santini et al (6) showed a significant decrease of circulating levels of VEGF in cancer patients with bone metastases receiving a single dose of pamidronate. Bezzi et al (7) showed that zoledronic acid inhibited endothelial cell adhesion and migration and enhanced tumor necrosis factor-mediated endothelial cell death. We previously demonstrated that neridronate inhibited angiogenesis in vitro and in vivo (8).

Herein, we examined the effects of clodronate in the early phases of the angiogenesis process, i.e. endothelial cell proliferation and in the late differentiative phases of neovascularization, i.e. formation of capillary-like structures by endothelial cells seeded on matrigel. In vitro observations were compared with the effects exerted in vivo by clodronate on physiological angiogenesis in the CAM assay, a useful model for such an investigation (9). The results demonstrate that clodronate inhibits angiogenesis in vitro and affects blood vessel formation in vivo.

Materials and methods

Cells and preparation of conditioned media (CM). Human umbilical vein endothelial cells (HUVECs) were prepared as previously described (10). The cells were grown in Petri dishes coated with 1% gelatin (Sigma Chemical Co., St. Louis, MO, USA) in a complete M199 medium (Seromed) supplemented with 20% heat-inactivated fetal calf serum (FCS) (Seromed), 0.02% bovine brain extract and 0.01% porcine heparin (both Sigma).

Proliferation assay. HUVECs (4x10⁴ cells/well) were plated in a 96-well plate (Falcon 3072, Becton Dickinson, Mountain...
Morphogenesis assay on matrigel. The assay assesses the ability of endothelial cells to produce ‘spontaneous angiogenesis in vitro’, i.e. a three-dimensional vascular tube and cord-like structure connecting ‘cellular nodes’ and resembling an organized capillary mesh. HUVECs cells (1.2x10^5/well) were plated in duplicate in 24-well plates (2x10^4 cells/well) precoated with matrigel (300 μl/well; Becton Dickinson) in 1 ml/well in a basal medium, containing or not FGF-2 (50 ng/ml) or clodronate at the same doses tested in the proliferation assay. After 18 h of incubation at 37°C, cell growth and tridimensional organization were observed through a reverted phase-contrast light microscope.

RESULTS

Effects of clodronate on the angiogenic phenotype of endothelial cells. The first series of experiments focused on the effects of clodronate on the proliferation of HUVECs. As shown in Fig. 1, clodronate reduced endothelial cell growth in a dose-dependent fashion peaking at 30 μM, whereas 50 μM gave a plateau.

In the second series, the effect of clodronate on capillary morphogenesis was investigated. After seeding on matrigel HUVECs in the presence of FGF-2 (50 ng/ml), HUVECs formed a rich meshwork of branching anastomosing capillary-like tubules with multicentric junctions (Fig. 2A). When FGF-2 was administered in the presence of clodronate (30 μM), the capillary-like tubes were interrupted and most cells were spherical, either isolated or aggregated in small clumps with only a few cells elongated (Fig. 2B).

Discussion

The existence of specific angiogenesis inhibitors was first postulated by J. Folkman in 1971 (12). The term ‘antiangiogenesis’ was introduced in order to describe treatment designed to prevent the induction of new blood vessels and perhaps to reduce the number of those already present.

Three strategies block tumor growth in experimental models through the regression of angiogenesis: vascular targeting, gene therapy and the direct inhibition of proliferating and migrating endothelial cells. Alternatively, indirect antiangiogenic drugs prevent expression or block the activity of tumor proangiogenic factors by interfering with their endothelial receptors.

Inhibitors of angiogenesis block any of the several steps in the angiogenic cascade. This includes proliferation and attachment of endothelial cells to the extracellular matrix proteins, migration and invasion through the matrix, which is required for the capillary sprouting and morphogenesis in a thin tube meshwork and differentiation and stabilization (13).

Several in vitro and in vivo studies indicate that bisphosphonates have antiangiogenic properties that could
contribute to their efficacy in the treatment of malignant bone
diseases and extend their potential clinical use to other
cancers and diseases with an angiogenic component, such as
inflammatory diseases.

We demonstrated that clodronate is an inhibitor of
angiogenesis in vitro, reducing the endothelial growth and
morphogenesis on matrigel. These in vitro effects were
matched by the inhibition of angiogenesis in vivo in the CAM,
where clodronate inhibited FGF-2-induced angiogenesis.

BP are potent inhibitors of bone resorption and are widely
used drugs for treatment of osteoporosis and bone metastasis
(14). In vitro anti-tumor properties of BP include inhibition of
tumor proliferation and invasion and induction of apoptosis of
various cancer cell lines, especially when combined with other
standard antineoplastic therapies (15). In vivo, this antitumor
effect appears to be limited to tumor cells in bone metastases,
at least in the majority of experiments performed to date (15).

BP has been reported to accumulate in human vessels
(16). Furthermore, nitrogen-containing BP such as ibandronate
and zoledronic acid have been shown to inhibit angiogenesis
in vitro and in vivo in several assays (3,16) or showed that
other BP antagonize angiogenesis and tumor growth in
traditional subcutaneous xenotransplant tumor models (17,18).

Yamagishi et al (19) demonstrated that minodronate, a
newly developed nitrogen-containing BP, can inhibit
melanoma growth and improve survival in nude mice by
suppressing the tumor-associated angiogenesis and
macrophage infiltration in vivo. Moreover, they also found that
minodronate blocked the angiogenesis signaling in
microvascular endothelial cells. Zeisberger et al (20) showed
that clodronate-liposome-mediated tumor associated macrophage depletion inhibited tumor growth, presumably through the blocking of tumor angiogenesis.

In recent years, studies based on human clinical models have been performed in order to confirm the antiangiogenic reports obtained by in vitro and in vivo preclinical studies (21).

Taken together, our results indicate that clodronate exerts its antiangiogenic activity through a direct effect on endothelial cell proliferative activity and an inhibitory effect on the responsivity of the endothelial cells to the proliferative stimuli mediated by an angiogenic cytokine, such as FGF-2. This suggests that clodronate, alone or in combination with other therapeutic strategies, may provide new opportunities to treat a wide spectrum of angiogenesis-dependent diseases, including certain chronic inflammatory diseases and cancer.

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References


