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# SELECTIVE INHIBITION OF CYSTEINE PROTEINASES BY Z-Phe-AlaCH$_2$F SUPPRESSES DIGESTION OF COLLAGEN BY FIBROBLASTS AND OSTEOCLASTS

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Effects of the selective inhibitor of cathepsins B and L, Z-Phe-AlaCH$_2$F were studied on the degradation of fibrillar collagen by fibroblasts and osteoclasts in cultured rabbit calvariae at the electron microscopic level. Periosteal fibroblasts from inhibitor-treated explants showed a dose-dependent increase of the volume fraction of vacuoles containing cross-banded collagen fibrils. This was a 7-fold increase over control fibroblasts and the ratio of intracellular and extracellular collagen increased from 2 to 43. The presence of collagen-containing vacuoles was also found in some osteoclasts from inhibitor-treated explants (1 $\mu$M or more). The inhibitor appeared to have cytotoxic effects at a concentration of 100 $\mu$M. It was concluded that this selective inhibitor exerts its effects intralysosomally in living cells, indicating possibilities for *in vivo* inhibition of protein degradation. 

Cysteine proteinases such as cathepsin B and L are involved in the intracellular degradation of phagocytosed collagen fibrils (1,2). Besides, they also seem to play a role in extracellular collagen degradation under pathological conditions such as cancerous growth and metastasis (3-11) and arthritis (12-15). Cysteine proteinases are involved in a number of other (patho)physiological processes among which lysosomal digestion of cytoplasmic proteins (16), liberation of thyroid hormone from thyreoglobulin (17,18), muscle protein turnover under normal conditions but also in muscular dystrophy (19), glomerulonephritis (20) and development of parasites such as *Plasmodium falciparum* in host erythrocytes (21).

A number of these observations are based on *in vivo* administration of more or less selective inhibitors of cysteine proteinases. However, most of these inhibitors have drawbacks for *in vivo* use such as reversibility of inhibition, lack of specificity and rapid clearance from the body. For instance, the microbial products antipain and leupeptin are effective but reversible inhibitors of cysteine proteinases (22). These compounds also inhibit certain serine proteinases (23). The compound E64 and its synthetic analogs are more selective inhibitors (24,25) but too quickly disappear from the circulation for *in vivo* use (26).

Peptidyl diazomethyl ketones are selective for cysteine proteinases: Z-Phe-PheCHN$_2$ inhibits cathepsin L but not B or H, whereas Z-Phe-AlaCHN$_2$ preferentially inhibits cathepsins B and L but not H (27-29). Their *in vivo* application is restricted since they

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appear to be mutagenic (30) and affect protein synthesis (31). The chloromethyl ketone counterparts are too electrophilic leading to indiscriminate alkylation *in vivo* (29). The peptidyl fluoromethyl ketone derivative Z-Phe-AlaCH$_2$F inhibits irreversibly and selectively cathepsins B and L and is 30-fold more potent than Z-Phe-AlaCHN$_2$ due to covalent modification of the cysteine residue in the active site of the proteinases (29,32). The compound is stable in the presence of thiol groups which rapidly destroy chloromethyl ketone derivatives and its reduced electrophilicity and high selective inhibitory capacity suggests possible therapeutic values for e.g. rheumatoid arthritis (14), tumor growth and metastasis (8) and infections with *Plasmodium falciparum* (21).

The present study was performed to investigate at the electron microscopical level effects of Z-Phe-AlaCH$_2$F on intracellular digestion of phagocytosed collagen fibrils by fibroblasts and the appearance of collagen-containing vacuoles in osteoclasts in cultured bone explants. Previous studies have shown that the protease inhibitors leupeptin and E64 inhibit lysosomal degradation of phagocytosed collagen fibrils by fibroblasts (1) and also results in the appearance of bone-derived collagen within lysosomal vacuoles of osteoclasts (2). These parameters were used to investigate the effectivity of the compound to inhibit intracellularly cathepsin B and L.

## MATERIALS AND METHODS

### Materials

Z-Phe-AlaCH$_2$F (32) was provided by Prototek (Dublin, CA, USA), Iscove's modified Dulbecco's medium (IMDM), normal rabbit serum, Fungizone$^R$ (amphotericin), streptomycin and penicillin were purchased from Gibco Labs. (Grand Island, NY, USA), dimethyl sulfoxide (DMSO) from Merck (Darmstadt, Germany), LX-112 resin from Ladd Research Industries (Burlington, VT, USA), and multi-well culture dishes from Costar (Cambridge, MA, USA).

### Culture methods

Calvariae from New Zealand White rabbits (1-2 weeks old) were dissected aseptically with surrounding periosteum. The calvariae were cut in small fragments (2x2 mm$^2$). Fragments were placed separately in wells of culture dishes and cultured for 24 h in media containing IMDM supplemented with 20% (v/v) normal rabbit serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 2.5 $\mu$g/ml amphotericin with or without 0.01-100 $\mu$M Z-Phe-AlaCH$_2$F dissolved in DMSO; the final concentration of DMSO in experimental as well as control wells was 0.03% (v/v). Incubations were carried out at 37°C in 5% $CO_2$ in air. After incubation, the explants were prepared for electron microscopy.

### Electron microscopy

Cultured calvarial explants were fixed for 24 h at 20°C in a solution containing 4% paraformaldehyde and 1% glutaraldehyde in 150 mM phosphate buffer (pH 7.4). After washing in 100 mM cacodylate buffer (pH 7.4) for 30 min at 4°C, the specimens were postfixed with 1% osmium tetroxide dissolved in 100 mM cacodylate buffer (pH 7.4) for 60 min at 4°C. The explants were dehydrated through a graded ethanol series and embedded in LX-112. Sections (1-2 $\mu$m thick) were cut and stained with Methylene Blue. The tissue blocks were trimmed and ultrathin sections were cut with a diamond knife. Sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope.

Morphometric analysis

From each explant one randomly selected ultrathin section was used for morphometric analysis.

Fibroblasts. Electron micrographs were taken of 10 to 20 fibroblasts in each of these sections. Starting at the edge of the section, micrographs were taken of periosteum at a distance of 8 $\mu$m from the surface of the bone and the distance between each consecutive micrograph was 16 $\mu$m. The micrographs were printed at a final magnification of x 23,340. The micrographs were coded, randomised and subjected to a point-counting procedure using a double lattice test grid with a 1:9 ratio (33). The surface densities of collagen-containing vacuoles (CCV), intracellular collagen fibrils (ICF), and extracellular collagen fibrils (ECF) were determined and expressed as percentage of fibroblast cytoplasm (1,34). CCV's were defined as membrane-bound profiles enclosing cross-banded collagen fibrils with the space between the membrane and the fibrils filled with electron-dense material. The lysosomal nature of these profiles was proven previously (1,35-38).

The data were subjected to statistical analysis using correlation analysis and Wilcoxon's two sample test ($2\alpha=0.05$).

Osteoclasts. Electron micrographs were taken of all cells exhibiting the morphological characteristics of osteoclasts (2,39,40). Osteoclasts were defined as large multinucleated cells containing a large number of mitochondria, free ribosomes, sparse endoplasmic reticulum, a ruffled border and a clear zone. The number of osteoclasts, with or without ruffled border, either or not adjacent to bone, the number of nuclei and the presence of collagen fibrils enclosed by the osteoclasts were determined (2).

## RESULTS AND DISCUSSION

The periosteal tissue surrounding the calvarial explants contained various cell types among which fibroblasts, osteoblasts, osteoclasts, macrophages and endothelial cells. The extracellular matrix consisted predominantly of loosely packed cross-banded collagen fibrils, the majority being orientated parallel to the surface of bone. The fibroblast was the most frequently occurring cell type and these cells were orientated parallel to the bone surface. They contained a well-developed rough endoplasmic reticulum and Golgi-apparatus and few mitochondria.

Treatment with 0.01-10 $\mu$M Z-Phe-AlaCH$_2$F did not seem to affect the morphology of the cells (cp Fig. 1 A & B). However, those incubated in the presence of 100 $\mu$M Z-Phe-AlaCH$_2$F appeared to have lost their long spindle-like shape, were rounded off, and nuclei were found to be pycnotic, indicating that the inhibitor is cytotoxic at a concentration of 100 $\mu$M.

Fibroblasts contained cytoplasmic vacuoles that were filled with cross-banded collagen fibril fragments. The fibrils were surrounded by either electron-dense or electron-lucent material. The number of electron-dense vacuoles containing fibrils was distinctly higher in fibroblasts treated with Z-Phe-AlaCH$_2$F (Fig. 1). Such vacuoles have been shown to be acid phosphatase-positive (1) suggesting their lysosomal nature. The collagen they contain

Fig. 1. Periosteal fibroblasts of calvarial explants cultured for 24 h in the absence (A) or presence (B) of 10 $\mu$M Z-Phe-AlaCH$_2$F. Arrows, vacuoles containing cross-banded collagen fibrils.

Fig. 2. Parts of osteoclasts adjacent to bone of explants cultured for 24 h in the absence (A) or presence of 10 $\mu$M (B, C) Z-Phe-AlaCH$_2$F. RB, ruffled border; B, bone; arrow, vacuolar-like structure containing cross-banded collagen fibrils; DB, demineralised bone; CZ, clear zone.

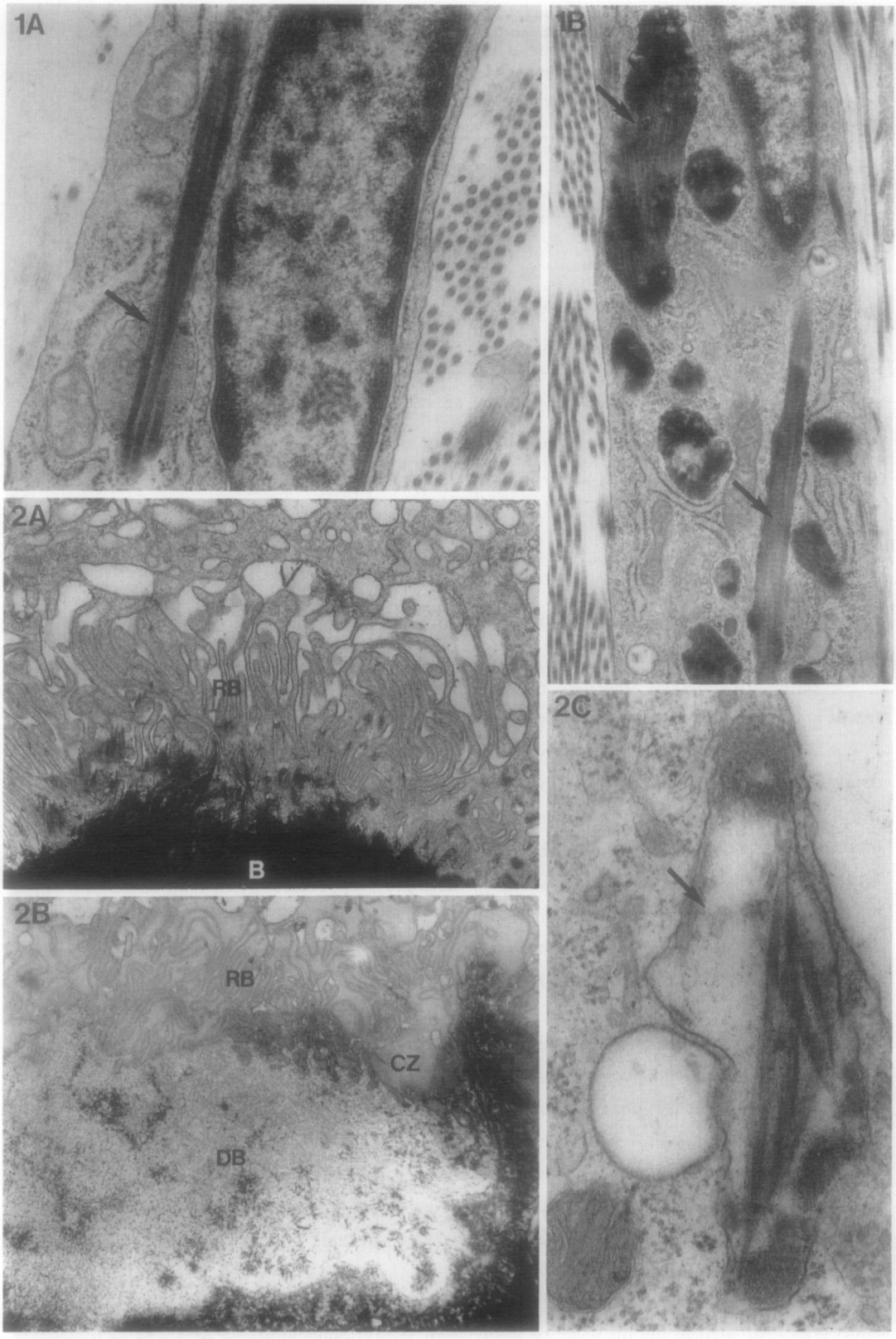
1A
1B
2A
V
RB
B
2C
2B
RB
CZ
DB

Table 1. Cytoplasmic volume densities of collagen-containing vacuoles (CCV) in periosteal fibroblasts and extracellular collagen fibrils (ECF) in periosteum and morphological parameters of osteoclasts after being cultured for 24 h in the presence or absence of 0.01-100 $\mu$M Z-Phe-AlaCH$_2$F (FMK)

| FMK ($\mu$M) | Fibroblasts | | | Osteoclasts | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | %CCV/Cyt. ±SD (a) | %CCV/ECF ±SD | n | Total number (b) | Adjacent (c) | Ruffled border (d) | (e) | Nuclei (f) | n |
| 0 | 0.23±0.11 | 0.22±0.12 | 6 | 32 | 21 | 13 (62%) | 0 | 2.5 | 6 |
| 0.01 | 0.64±0.38 | 0.70±0.43 | 6 | 33 | 21 | 6 (29%) | 0 | 2.1 | 6 |
| 0.1 | 0.62±0.20 | 0.90±0.06 | 6 | 13 | 12 | 3 (25%) | 0 | 2.6 | 6 |
| 1.0 | 1.28±0.62 | 2.26±1.25 | 6 | 34 | 25 | 3 (12%) | 3 | 2.3 | 6 |
| 10 | 1.52±0.47 | 4.25±2.51 | 6 | 32 | 23 | 8 (35%) | 3 | 2.2 | 6 |
| 100 | 1.10±0.71 | 1.89±1.51 | 6 | 39 | 23 | 2 ( 9%) | 2 | 2.2 | 6 |

(a) percentage CCV of total cytoplasm of fibroblasts
(b) number of osteoclasts
(c) number of osteoclasts attached to bone
(d) number of osteoclasts with a ruffled border; also given between brackets as percentages of (b)
(e) number of osteoclasts with vacuole-like structures containing cross-banded collagen fibrils
(f) mean number of nuclei per osteoclast per section
n number of calvariae

represents phagocytosed collagen fibrils and not accumulated newly synthetised collagen (1,34).

Morphometric analysis revealed a dose-dependent increase up to 10 $\mu$M of the volume fraction of vacuoles containing fibrils ($p<0.025$; Table 1). At a concentration of 10 $\mu$M Z-Phe-AlaCH$_2$F, 15% of the fibroblast cytoplasm was occupied by electron-dense vacuoles containing cross-banded collagen fibrils which amounts to a 7-fold increase over control fibroblasts. The ratio of intracellular and extracellular collagen showed a dose-dependent increase up to 10 $\mu$M Z-Phe-AlaCH$_2$F ($p<0.025$) from approx. 2 to 43 (Table 1). These findings demonstrate that Z-Phe-AlaCH$_2$F strongly suppresses lysosomal digestion of phagocytosed collagen by fibroblasts. These observations are in line with similar studies on the effects of leupeptin and E64 (1) and confirm that cathepsin B and/or L play a crucial role in the intracellular digestion of phagocytosed collagen.

Osteoclasts showed a number of morphological changes upon treatment with Z-Phe-AlaCH$_2$F (Fig. 2). Cells from explants cultured in the presence of the highest concentration of the inhibitor contained pycnotic nuclei, but the number of nuclei found per osteoclast did not change significantly upon treatment (Table 1). Pycnotic nuclei were not found in osteoclasts treated with 0.01-10 $\mu$M Z-Phe-AlaCH$_2$F. The percentage of osteoclasts that were in apposition to bone did not vary upon treatment (approx. two-thirds of the osteoclasts were found to be in close apposition to bone in all periostea), but the percentages of these cells that showed a ruffled border was 62% in untreated explants and at most 35% in treated explants (Table 1).

Vacuole-like structures containing cross-banded collagen fibrils were found solely in osteoclasts incubated in the presence of Z-Phe-AlaCH$_2$F at concentrations of 1 $\mu$M or higher.

At these concentrations, such structures were found in 8 out of 13 osteoclasts with a ruffled border (Fig. 2B). Most of these vacuole-like structures were electron-translucent suggesting that collagen was surrounded by the osteoclasts but not actually internalised (2). These findings are similar to data previously published on the effects of leupeptin and E64 on osteoclastic bone digestion (2), and may suggest that non-degraded demineralised bone collagen fibrils are (in the process of being) taken up by osteoclasts under the influence of Z-Phe-AlaCH$_2$F. However, since the total number of osteoclasts in the present study was rather low - possibly due to the fact that parathormone had not been added to the culture medium - further studies are needed to analyse the effects of the inhibitor on bone digestion. A second effect of leupeptin and E64 which has been found previously (2) was the appearance of large demineralised areas of bone adjacent to the osteoclasts. In the present study, demineralised bone adjacent to a ruffled border was observed only infrequently: a single osteoclast incubated in the presence of 0.1 $\mu$M and three osteoclasts incubated in the presence of 10 $\mu$M Z-Phe-AlaCH$_2$F showed signs of demineralisation of the underlying bone (Fig. 2C). These limited effects of Z-Phe-AlaCH$_2$F on demineralisation may also be due to the fact that bone resorption had not been stimulated in the cultures by addition of parathormone.

The effect of Z-Phe-AlaCH$_2$F appeared to be maximal at a concentration of 10 $\mu$M (Table 1), whereas leupeptin and E64 exert their maximum effects at concentrations of 325 and at least 100 $\mu$M, respectively (1). Although 100 $\mu$M Z-Phe-AlaCH$_2$F showed indications of cytotoxic effects on fibroblasts and osteoclasts, which has never been observed with leupeptin and E64, the *in vivo* toxicity is low ($LD_{50}$ = 360 mg/kg in mice (29)). The significant effects of Z-Phe-AlaCH$_2$F at rather low concentrations on fibroblasts and osteoclasts warrant further investigations on the *in vivo* use of peptidyl fluoromethyl ketones for modulation of various disorders in which proteases are involved. The more so, because the fluoromethyl ketones are irreversible inhibitors. The present study also underlines the usefulness of this type of inhibitor for the elucidation of the involvement of the different proteases in cell biological processes in general.

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