ORIGINAL ARTICLE

METABOLISM OF THE COMPLEX MONOFLUOROPHOSPHATE-α2-MACROGLOBULIN IN THE RAT

LUIS ESTEBAN, ALFREDO RIGALLI, RODOLFO C. PUCHE

Laboratorio de Biología Osea, Facultad de Ciencias Médicas, Universidad Nacional de Rosario

Abstract   Sodium monofluorophosphate (MFP) is a drug used in the treatment of primary osteoporosis. Following the intake of MFP, a small fraction of the drug is absorbed intact and forms a complex with α2-macroglobulin (MFP-α2M) inactivating the antiproteasic activity of the globulin. The complex has been shown to occur in the serum of rats and human being. This paper reports data on the metabolism of this complex in the rat. In vitro experiments showed that liver and bone tissue remove MFP-α2M from the incubation medium. When the experiments were pursued beyond the time needed to reduce the complex concentration to very low levels, fluorine (F) reappears in the medium in two forms: bound to low molecular weight macromolecule/s (2,200 ± 600 Da) and as ionic F. Concentrations of these F fractions increase while that of the complex decreases as a function of time. In vitro, uptake of the complex by liver or bone tissue was not affected by the presence of colchicine or methylamine. These drugs, however, inhibited intracellular metabolism of the complex, as indicated by the impairment of the return of F species to the extracellular space and the increase in F content of the tissue. The cellular receptors responsible for the uptake of the complex in liver and bone are insensitive to low concentration of calcium and inhibited by polyinosinic acid[5']. These features characterize the "scavenger" receptor, one of the two receptor types known to remove inactive α2M from the circulation. Injection of polyinosinic acid [5'] to living rats also hindered the disappearance of the complex from serum. It is concluded that the metabolism of the MFP-α2M complex involves binding to receptors, uptake by cells, lysosomal degradation and return of F bound to low molecular weight macromolecule/s to the extracellular space. It is assumed, however, that inorganic F is the final product of lysosomal hydrolysis of the protein moiety.

Resumen   *Metabolismo del complejo monofluorfosfato-α2-macroglobulina en la rata.* El monofluorfosfato de sodio (MFP) es una de las drogas empleadas para el tratamiento de la osteoporosis. Después de cada dosis de MFP, una fracción de la misma se absorbe intacta, forma un complejo con la α2-macroglobulina del plasma (MFP-α2M) que provoca la pérdida de la actividad antiproteásica de la globulina. Este trabajo describe el metabolismo del complejo MFP-α2M. Experimentos in vitro demostraron que los tejidos óseo y hepático extraen el complejo MFP-α2M del medio de incubación. Cuando los experimentos se prolongaron más allá del tiempo necesario para reducir la concentración del complejo a niveles insignificantes, el flúor reapareció en el medio de incubación en dos formas: ligado a moléculas de bajo peso molecular (2 200 ± 600 Da) y como flúor iónico. Las concentraciones de estas fracciones aumentaron en el medio de incubación en función inversa a la concentración del complejo. El clearance del complejo MFP-α2M, efectuado in vitro por los tejidos óseos y hepático, no fue afectado por la presencia de colchicina o metilamina. Estas drogas inhibieron el metabolismo intracelular del complejo, indicado por el aumento del contenido tisular de flúor y la ausencia del fenómeno de circulación de especies de flúor al medio de incubación, aludido más arriba. Los receptores celulares responsables del clearance del complejo son del tipo "scanverger": insensibles a bajas concentraciones de ión calcio e inhibidos por ácido [5']poli-inosínico. Se concluye que el metabolismo del complejo MFP-α2M involucra ligamiento a receptores de membrana, incorporación a las células, degradación lisosomal y retorno al espacio extracelular de especies de flúor, de peso molecular progresivamente más bajo, hasta flúor iónico. Se atribuye la mayor biodisponibilidad de flúor del MFP (respecto del NaF), a los fenómenos descriptos.

Key words: monofluorophosphate, α2-macroglobulin, α2-macroglobulin-monofluorophosphate complex, (ionic) serum F, (protein bound) serum F

Sodium monofluorophosphate (MFP) is a drug used in the treatment of primary osteoporosis[1, 2]. It has greater gastric tolerance than NaF and it can be administered with calcium salts[3, 4]. After an oral dose of MFP, a fraction of the dose is absorbed without hydrolysis and binds to

plasma proteins[5], a phenomenon observed in rats[5, 6] and human beings[7]. MFP binds to α2-macroglobulin (α2M) and to C3 (a member of the complement system) with the loss of activity of these proteins[8].

This protein-bound fluorine (F) compartment assumedly explains the greater bioavailability of F from MFP than from NaF[6, 7].

This paper investigates the metabolism of MFP-α2-macroglobulin complex (MFP-α2M) in the rat. The data obtained show that liver and bone actively remove the

complex from the extracellular space. F returns to the latter space as ionic F and bound to low molecular weight macromolecule/s.

Material and Methods

Animals. Female rats IIM/Fm strain, substrain "m"[9], were employed in all experiments. "In vivo" experiments were carried out with seven weeks old rats (200 ± 21 g, mean ± SEM). Liver and bone tissue (parietal bones) were obtained from 7 and 3 weeks old rats (30 ± 5 g), respectively.

Reagents. MFP was obtained from Ozark Mahoning, Tulsa, OA USA. The drug employed in these experiments contained 96.5% of the theoretical amount of MFP and 3.5% of ionic F, measured as shown elsewhere[5].

α2-macroglobulin (α2M), colchicine, methylamine, EDTA and polyinosinic [5'] acid were purchased from Sigma Chemical Co. (St. Louis, MO). Other drugs were analytical grade.

Preparation of MFP-α2M. α2M was isolated from human (citrated) plasma as reported by Swenson and Howard[10] and dissolved in Krebs Ringer Bicarbonate buffer (KRB) to attain a concentration of 40 g/l (0.2 mM). Human plasma was obtained at the blood bank of the University Hospital.

MFP was added to α2M solution to attain a 1 mM concentration and incubated for 30 min at 20°C[8]. The mixture was then chromatographed on a column (110 ml volume, 80 cm length) filled with Sephadex G-100 (Pharmacia, Uppsala, Sweden) suspended PBS (saline containing 50 mM phosphate, pH 7.4). MFP-α2M eluted in the first 25-30 ml while free MFP and traces of ionic F eluted together at 105-110 ml. According to previous studies[8], the MFP-α2M complex contains 1 mole of F per mole of α2M.

Molecular weight estimation. A column (20 ml volume, 17 cm length) was filled with Sephadex G-50 (Pharmacia, Uppsala, Sweden) suspended PBS (saline containing 50 mM phosphate, pH 7.4). The column was loaded with 0.2 ml aliquots of plasma or incubation medium. Elution was performed with PBS. Forty fractions of 0.5 ml were collected. Protein concentration was monitored by UV absorption at 280 nm and total F was measured as said below.

The column was calibrated with a mixture of oxytocin (1000 Da), C peptide (3000 Da) and insulin (6000 Da) for molecular weight estimation. K_{av} was plotted against molecular weights (Figure 4). K_{av} was calculated as the ratio $(V_e-V_o)/(V_t-V_o)$ where V_e, V_o and V_t stands for elution, void and column volumes, respectively.

Measurement of fluorine (F)

Total F (protein bound + ionic) content of plasma, urine, chromatography fractions, tissue, etc., was accomplished by means of the isothermic distillation method of Taves[11].

Diffusible F. Plasma or incubation buffer (1.5-2 ml) were ultrafiltered through Centriflo membranes (Amicon Corp., Lexington, MA, USA), with a 25000 molecular weight cut-off. F was measured in the ultrafiltrate by the method of Taves[11]. The reader should note that besides ionic F, this fraction may contain low molecular weight protein-bound F.

The difference between total and diffusible F is assumed to measure MFP-α2M.

F was measured in the Taves' distillate with an ion-specific electrode (94-09 Orion Research Inc., Cambridge, MA, USA) after samples were treated as stated above. Electrodes were assembled as indicated by Hallsworth et al.[12]

In vitro studies

The tissues employed (liver or bone) were excised, rinsed twice with KRB at 4°C and cut into small pieces (0.5 mm), by hand, with scalpels.

Incubation of tissue (liver: 20-25 mg/4 ml of KRB, bone: 90-100 mg/4 ml) was done in plastic tubes, at 37°C. Shaking was accomplished by a gentle stream of carbogen (95% O_2-5% CO_2). MFP-α2M was added to KRB to attain a concentration 40-50 µM. In experiments designed to measure only the rate of disappearance of MFP-α2M from the incubation medium, aliquots were removed at 0, 2, 7.5, 15, 20 and 30 minutes, centrifuged and kept frozen until F analysis. The data obtained fitted first order kinetics ($C = C_o \exp\text{-}k_e t$), where C_o is the initial concentration, C that at time t and ke is the rate constant (min^{-1}) of disappearance of MFP-α2M from the incubation medium.

Some experiments were done to investigate the return of F to the extracellular space. Aliquots were removed at 0, 2, 7.5, 15, 30, 60 and 90 minutes, centrifuged and kept frozen until F analysis. In parallel experiments, the incubation media were chromatographed on Sephadex G50 (see above, Molecular weight estimation). Total F content of fractions was determined in each fraction.

Minced tissue was treated with the following additions to the incubation medium, as indicated below: EDTA (4.7 mM), methylamine (65 mM), or polyinosinic acid[5'] (250 µg/ml). Colchicine treatment was done as follows: tissue was incubated during one hour in KRB with the drug (10 µM), recovered by centrifugation at low speed and reincubated in KRB+α2M-MFP. The rationale for the use of these drugs is explained in the Discussion section. Briefly, EDTA and polyinosinic acid[5'] were used to characterize the type of membrane receptor for α2M-MFP; methylamine and colchicine inhibit at different points the process of internalization and proteolysis of the complex receptor-α2M-MFP.

At the end of the experiments with colchicine and methylamine, control and treated tissue were recovered by centrifugation and assayed for total F. Tissue content of F is expressed as µmol/g of wet tissue.

In vivo experiments

Rats (220 ± 21 g) fed *ad libitum* with standard chow were used in the following experiments. These were designed to investigate the clearance of MFP-α2M, to verify the inhibitory effect of polyinosinic acid[5'] on that variable, and to determine the F species of rat serum after an oral dose of MFP. Two types of experiments were done:

1) after intravenous injection of the complex.

The rats were hydrated with 15 ml of tap water administered by gastric tube to insure a significant urine flow. Anaesthesia was done by intraperitoneal injection of 120 mg urethane/100 g body weight. The left femoral vein was catheterized to facilitate iv injections of the complex or of polyinosinic acid or to obtain blood samples. Urine was collected through a vesical catheter.

The animals received MFP-α2M (0.1 µmoles/100 g bw) by iv injection. Some rats received polyinosinic acid [5'] (5 mg in 0.2 ml of PBS/EDTA, pH = 7.4)[13], one minute before the injection of the MFP-α2M complex. Urine was collected during 15 minu-tes before the injection of the complex and during the rest of the experiment. At the times indicated in Figure 5 (left panel), 0.2 ml blood samples were drawn from the catheter inserted into the femoral vein. The samples were centrifuged 5 min at 8000 g and aliquots of plasma were separated and frozen at -20°C for F assays (total and diffusible).

The difference between plasma total and diffusible F was assumed to measure MFP-α2M. The data fitted the same function (C = C_o exp -k_et) described above. The rate constant of MFP-α2M dissappearance from plasma (k_e, min^{-1}) was calculated using a computer program[14].

2) after a single oral dose of MFP.

The animals were anesthetized as said above. Four rats received the injection of 5 mg of polyinosinic acid [5'] dissolved in 0.2 ml of PBS/EDTA, pH = 7.4, through a catheter placed in the femoral vein, fifty minutes after a dose of 40 μmol MFP/100 g b.w by gastric intubation. Control rats (n = 4) received only the oral dose of MFP. At the times indicated in Figure 5 (right panel), aliquots of blood samples were taken and processed as stated above.

Calculation of the rate constant was done with the plasma values obtained from 60 minutes onwards, because when serum MFP-α2M reaches its maximum value after the oral adminis-tration of MFP[6]. The data fitted the same function (C = C_o exp -ke_et) described above. The rate constant of disappearance of MFP-α2M from the circulation (k_e, min^{-1}) was calculated using a computer program [14].

Two hours after the oral administration of MFP, the animals were sacrificed by exsanguination. Plasma was saved for chromatography on Sephadex G50 as indicated above to determine the presence of F bound to peptide/s and their molecular weight.

Statistical techniques

Regression analysis and Student's "t" test for grouped and paired data were employed for evaluation of the data[15].

Results

In vitro experiments

Liver and bone tissue removed the complex MFP-α2M from the incubation medium (Figures 1 and 2, Table 1). As discussed below, these results suggested the existence of receptors responsible for the removal of the complex from the extracellular space. Some of these experiments were done with human α2M (instead of rat α2M)

because it is known that inactive α2M is recognized by receptors present in rat tissue[16].

Some experiments were designed to identify the type of receptor responsible for the removal of MFP-α2M from the incubation medium. The phenomenon was strongly hindered by polyinosinic acid[5'] but not affected by very low levels of Ca^{++} (EDTA addition) (Figure 2, Table 1). As discussed below, these results suggested that the "scavenger" type of receptor (sensitive to polyinosinic acid[5'], insensitive to Ca^{++})[13].

When liver or bone tissue were incubated beyond the time needed to remove MFP-α2M from the incubation buffer, F returned to the extracellular space (Figure 1). In these experiments, extracellular F eluted from Sephadex G50 columns in three fractions (Figure 3). The first, eluting with a peak at 1-2 ml, is the complex MFP-α2M. A

TABLE 1.– *Experiments in vitro. Rate constants of the removal of MFP-α2M from the incubation medium*

Tissue	Addition	Rate constant min^{-1}	P
Liver	none	0.29 ± 0.04	
	EDTA	0.28 ± 0.08	0.916
Bone	none	0.49 ± 0.02	
	EDTA	0.41 ± 0.14	0.589
Liver	none	0.28 ± 0.06	
	Polyinosinic	0.06 ± 0.05	0.036
Bone	none	0.42 ± 0.11	
	Polyinosinic	0.10 ± 0.03	0.031
Liver	none	0.27 ± 0.09	
	Colchicine	0.28 ± 0.15	0.789
Liver	none	0.25 ± 0.10	
	methylamine	0.27 ± 0.12	0.885

The figures indicate the mean ± SEM (N = 4)
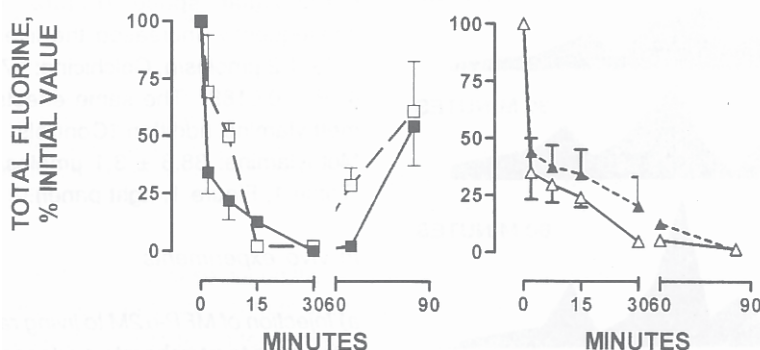

Fig. 1.– Left panel: change of MFP-α2M concentration in the incubation medium as a function of time. Experiments with liver and bone are indicated by solid and open squares, respectively. Bars indicate SEM of the mean (n = 4). Right panel: change of MFP-α2M concentration in the incubation medium with liver tissue treated with colchicine (solid triangles) and methylamine (open triangles). Bars indicate SEM of the mean (n = 4).
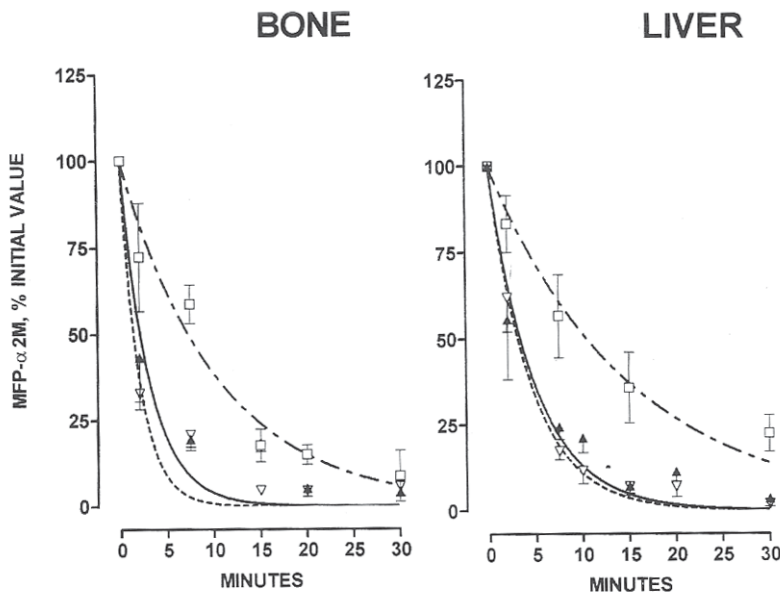
BONE

LIVER

Fig. 2.– Uptake of MFP-α2M complex in vitro by bone (left panel) and liver tissue (right panel) Controls: solid line and solid triangles, EDTA: dashed line and open triangles, polyinosinic acid [5']: dashes and points line and open squares. Bars indicate SEM of four experiments. Curves were derived with a computer; rate constants are listed in Table 1.
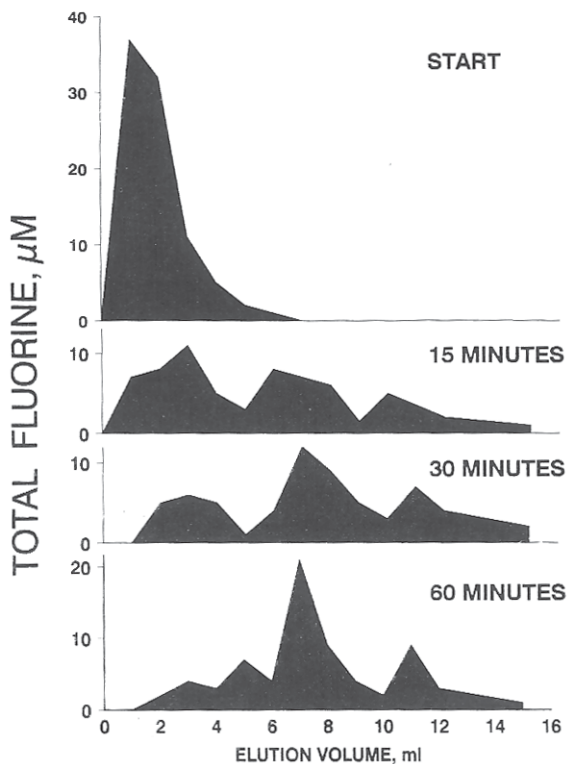

Fig. 3.– Liver tissue incubated with MFP-α2M. Elution patterns of total F of the incubation medium chromatographed on Sephadex G50, at the start and 15, 30 and 60 minutes later.

second fraction, eluting at 6-7 ml, is F bound to low molecular weight macromolecule/s (2200 ± 600 Da) (Figure 4). The third, with a peak at 10-12 ml, reacted directly with the ion selective electrode, and had the same elution pattern as ionic F. The disappearance of MFP-α2M and appearance of ionic fluoride and F bound to low molecular weight macromolecule/s is a function of time (Figure 3).

Treatment of the tissue with colchicine (10 µM) did not affect the removal of MFP-α2M from the incubation medium (Table 1) but inhibited recirculation of F to the extracellular space (Figure 1, right panel) and consequently, increased the liver F content (Controls: 7.7 ± 1.2 µmoles/ g, Colchicine: 17.3 ± 3.4 µmoles/g, N = 4, P = 0.0188). The same effects were observed after methylamine addition (Controls: 10.0 ± 1.5 µmoles/g; Methylamine: 38.8 ± 3.1 µmoles/g, N = 4, P < 0.001) (Table 1, Figure 1, right panel).

In vivo experiments

a) Injection of MFP-α2M to living rats. These experiments were done to corroborate evidence obtained in vitro. After iv injection of MFP-α2M, the rate constant for removal of the complex from the circulation had a significant value in control rats ($Ke = -0.057 \pm 0.0028$ min^{-1}, P = 0.0481). In the animals that received 5 mg of polyinosinic acid[5'] by intravenous injection (Figure 5, left panel) the constant did not differ from zero (-0.015 ± 0.0071 min^{-1}).
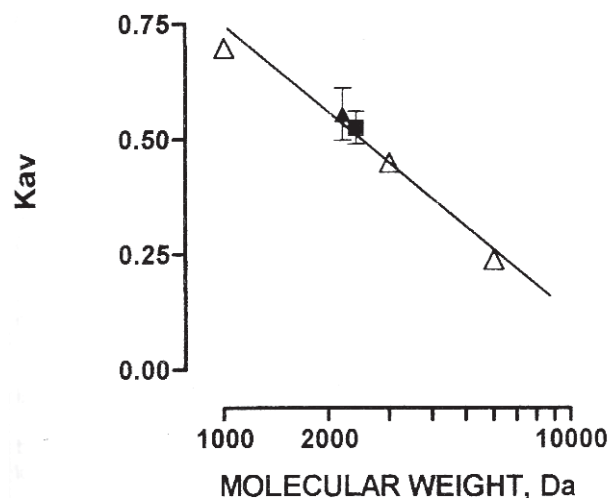
Fig. 4.– Molecular weight estimation of the macromolecule/s eluted with a peak at 6-7 ml from the column of Sephadex G50. Experiments in vitro (liver and bone tissue): solid triangle ± SEM of four experiments. Experiments in vivo (plasma of rats after an oral dose of MFP): solid square ± SEM of four experiments. Standards (open triangles) were, from left to right: oxytocin, C-peptide and insulin.
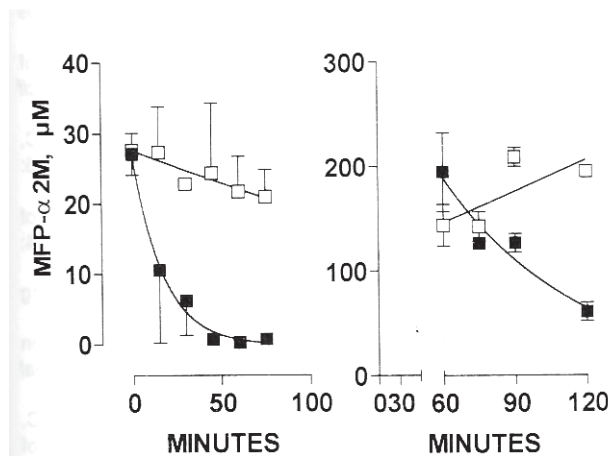

Fig. 5.– Removal of MFP-α2M from rat serum, in vivo. The complex was injected intravenously (left panel, solid squares) or it was produced by oral administration of MFP to the animals (right panel, solid squares). Open squares show the data of animals that also received polyinosinic [5'] acid. Bars indicate the SEM of 4 experiments.

The urinary excretion of F did not increase during these experiments. Basal urinary F excretion of controls was (mean ± SEM) 51 ± 10 nmoles F/90 minutes; after iv injection of MFP-α2M: 42 ± 9 nmoles/90 minutes, N = 4, P > 0.05. In polyinosinic[5'] acid treated rats, basal F excretion was 55 ± 18 nmoles/90 minutes; after iv injection of the complex: 48 ± 9 nmoles/90 minutes, N = 4, P > 0.05.

b) Clearance of MFP-α2M formed after the oral intake of MFP. We have shown elsewhere[5, 6] that after an oral

dose of MFP, the complex appears in plasma with maximum level 60 minutes after dosing. The rate constant for the removal of MFP-α2M from plasma was (mean ± SEM) -0.018 ± 0.003 min^{-1}, P = 0.0186. The constant mesured in animals that received 5 mg of polyinosinic acid [5'] did not differ from zero: 0.0010 ± 0.0051 min^{-1} (Figure 5, right panel).

The observed rate constant for the removal of the MFP-α2M complex formed in vivo in the rat (-0.018 min^{-1}) is 3 times lower than that observed after iv injection of the complex preformed in vitro with human α2M (-0.057 min^{-1}). The data may reflect differences in the metabolism of the complexes MFP-a2M (rat) and MFP-a2M (human) by the living rat. These experiments, however, are qualitatively similar: polyinosinic acid [5'] interferes in vivo with the serum clearance of the complex MFP-α2M.

Discussion

The presence of the complex monofluorophosphate-α2-macroglobulin (MFP-α2M) has been shown previously in the sera of rats and human beings[8], after an oral dose of MFP. MFP and α2M react spontaneously at room temperature. The bond is stable. Its nature and the binding site, however, are still unknown[8].

The complex MFP-α2M shares two features with other α2M complexes (e.g.: trypsin-α2M): loss of antiproteasic activity and its rapid removal of the complex from circulation[8]. With one exception, the experiments reported in this paper were done with α2M isolated from human plasma. Human α2M is recognized by receptors present in rat liver[16]. To verify whether the receptor described in liver was also present in bone tissue, complementary experiments were done with the latter.

The receptor for MFP-α2M in rat liver and bone tissue exhibits the features reported for the "scavenger" receptor: it is sensitive to polyinosinic acid[5'] and indiferent to low Ca^{++} levels[13]. It is believed that its presence in bone has physiological significance because MFP is used to increase bone mass through activation of osteoblasts. Present data do not allow to compare tissue scavenging activities. The reader should note the differences in the age of donor animals and in the number of cells of both tissues per unit of wet weight.

Removal of MFP-α2M from the extracellular space is shown to occur both in vitro and in vivo. In vitro, the phenomenon is initially so rapid (75% of initial concentration disappears from the incubation medium in 5-7 minutes) that it is assumed to be the sum of binding to receptors and (the energy-dependent) uptake by the cells. This assumption is supported by the data obtained from 0 to 30 minutes, both panels of Figure 1. Methylamine of colchicine (see below) did not interfere with binding or uptake of the complex.

Receptor-mediated internalization and degradation of the protein has been described for all receptor bearing cell types. The data obtained fits the general scheme gained on α2M metabolism (reviewed in[17]). Immediately after ligand binding, the α2M receptors that are initially scattered over the cell surface, begin to aggregate over clathrin coated areas of the membrane. This aggregation is necessary for internalization of the receptor-ligand complex. Coated regions rapidly become invaginated into so-called coated pits and within minutes, ligand and receptors can be located in intracellular coated vesicles that are connected with the cellular surface only by a tubular neck. Inhibitors of this process, antitubulins like colchicine[18], downregulate uptake and degradation of α2M. Within minutes, α2M accumulates in the lysosomes where it is degraded. The degradation products are rapidly ejected from the cells and appear extracellularly with a short lag time (minutes) corresponding to the time it takes for internalized α2M to reach the lysosomes, indicating almost instant lysosomal degradation. Weak and highly diffusible bases like methylamine that may neutralize pH of uncoated vesicles[19], are potent inhibitors of both ligand degradation and the reappearance of α2M receptor in the surface membrane. The data shown in the Figures and in the Table 1, fully agree with this description.

In vivo, the rate constant for disappearance of the complex is three times greater with α2M of human origin than that measured with the complex formed sponta-neously in rat plasma after an oral dose of MFP. These results are polyinosinic acid[5'] probably related to species differences in α2M. Both *in vivo* and *in vitro*, polyinosinic acid[5'] affects adversely the rate constants for the disappearance of MFP-α2M from the extracellular space.

The urinary excretion of ionic fluoride was not affected in short term experiments (90 minutes), after the iv injection or its endogenous formation. These findings suggest that the ionic F produced by degradation of the complex in 90 minutes is insufficient to affect the F basal urinary excretion. In 7-hour experiments published elsewhere[6], however, the urinary excretion of fluoride increased over basal levels: 9.8 ± 1.2 (N = 6) vs 0.2 ± 0.09 (n = 12) µmoles/7 hours.

It is concluded that the metabolism of the MFP-α2M complex follows the pathways: uptake through the scavenger receptor, lysosomal degradation and return of F bound to low molecular weight macromolecule/s. Inorganic F is the final product of peptide degradation.

These phenomena contribute to explain the greater F bioavailability of MFP (compared with NaF), shown elsewhere by pharmacokinetic studies in rats[6] and human beings[7].

References

1. Delmas PD, Dupuis J, Duboeuf F. Treatment of vertebral osteoporosis with disodium monofluorophosphate: comparison with sodium fluoride. *J Bone Min Res* 1990; 5 (Suppl. 1): S143-S147.
2. Sebert JL, Richard PR, Mennecier I, Bisst JP, Loeb G. Monofluorophosphate increases lumbar bone density in osteopenic patients: a double-masked randomized study. *Osteoporosis Int* 1995; 5: 108-14.
3. Ericsson YT. Monofluorophosphate physiology: General considerations. *Caries Res* 1983; 17 (Suppl 1): 46-55.
4. Ericsson Y, Santesson G, Ellbes J. Absorption and metabolism of the PO4F in the animal body. *Arch Oral Biol* 1961; 4: 160-74.
5. Rigalli A, Cabrerizo MA, Beinlich AD, Puche RC. Gastric and intestinal absorption of monofluorophosphate and fluoride in the rat. *Arzneim Forsch/Drug Res* 1994; 44:651-5.
6. Rigalli A, Ballina JC, Beinlich A, Alloatti R, Puche RC. Pharmacokinetic differences between sodium fluoride and sodium monofluorophosphate and comparative bone mass increasing activity of both compounds in the rat. *Arzneim Forsch/Drug Res* 1994; 44: 762-6.
7. Rigalli A, Morosano M, Puche RC. Bioavailability of fluoride administered as sodium fluoride or sodium monofluoro-phosphate to human volunteers. *Arzneim Forsch/Drug Res* 1996, 46: 531-3.
8. Rigalli A, Esteban L, Pera L, Puche RC. Binding of monofluorophosphate to α2-macroglobulin and C3. *Calcif Tissue Int* 1997; 60: 86-9.
9. Font MT, Garroq O, Martinez SM, Morini JC, Puche RC, Tarreas MC. *The inbred II M/Fm stock rat Newslt* 1991; 25: 28-9.
10. Swenson RP, Howard JB. Structural characterization of human α2-Macroglobulin subunits. *J Biol Chem* 1979; 254: 4452-6.
11. Taves D. Separation of F by rapid diffusion using hexametildisiloxane. *Talanta* 1968; 15: 969-74.
12. Hallsworth AS, Weatherell JA, Deutsch D. Determination of subnanogram amounts of fluoride with electrode. *Anal Chem* 1976; 48: 1660-4.
13. Van Dijk MCM, Boers W, Linthorst C, Van Berkel TJC. Role of the scavenger receptor in the uptake of methylamine inactivated α2-macroglobulin by rat liver. *Biochem J* 1992; 287: 447-55.
14. Tallarina R, Murray R. Manual of Pharmacologic Calculations With Computer Programs. 2nd Edition. New York: Springer-Verlag 1986.
15. Snedecor GW, Cochram WG. Statistical Methods, 6th. ed. Ames, IA: Iowa State University Press 1967.
16. Davidsen O, Christensen ET, Gliemann J. The plasma clearance of human α2-macroglobulin-trypsin complex in the rat is mainly accounted for by uptake into hepatocytes. *Biochim Biophys Acta* 1985; 846: 85-92.
17. Petersen CM. α2-macroglobulin and pregnancy zone protein. *Danish Med Bull* 1993; 40: 409-46.
18. Aihao H, Ding C, Porteau F, Sanchez E, Nathan C. Downregulation of tumor necrosis factor receptors on macrophages and endothelial cells by microtubule depolymerizing agents. *J Exp Med* 1990; 171: 715-27.
19. Tycko B, DiPaola M, Yamashiro H, Darrel J, Fluss S, Maxfield Fr. Acidification of endocytic vesicles and the intracellular pathways of ligands and receptors. *Ann NY Acad Sci* 1983; 421: 420-8.