Agmatine Suppresses Mesangial Cell Proliferation by Modulating Polyamine Metabolism

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ETO, S., ISOME, M., SANO, H., FUKUDA, Y., KAWASAKI, Y., SUZUKI, J., IGARASHI, K., SATRIANO, J. and SUZUKI, H. *Agmatine Suppresses Mesangial Cell Proliferation by Modulating Polyamine Metabolism.* Tohoku J. Exp. Med., 2006, **210** (2), 145-151 — Polyamines play an essential role in the growth and differentiation of mammalian cells. The depletion of intracellular polyamines results in the suppression of growth. Proliferation of glomerular mesangial cells (MC) is the most common pathologic change in many forms of glomerulonephritis. Agmatine is a metabolite of arginine via arginine decarboxylase (ADC), highly expressed in the kidney, and unique in its capacity to suppress intracellular polyamine levels required for proliferation. As agmatine enters mammalian cells via the polyamine transport system, its antiproliferative effects may preferentially target cells with increased proliferative kinetics. In the present study, we evaluated the antiproliferative effects of agmatine on human MC in vitro. MC proliferation was stimulated with 20% fetal bovine serum (FBS) or platelet-derived growth factor (PDGF-BB, 20 ng/ml). Cell proliferation was measured using the (4,3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) proliferation assay. Intracellular polyamine levels were assayed by high performance liquid chromatography, and cell death was assessed by cellular DNA fragmentation enzyme-linked immunosorbent assay. The MTT proliferation assay showed that agmatine significantly suppressed proliferation of human MC treated with 20% FBS or 5% FBS + PDGF as compared to human MC treated with 5% FBS. Polyamine levels were markedly lower in cells treated with agmatine, and proliferation was rescued by administration of putrescine. The fragmented DNA was hardly detected in agmatine-treated human MC. In summary, human MC stimulated to increase their proliferative kinetics are significantly more sensitive to the antiproliferative effects of agmatine than normally cultured cells. Suppressed proliferation of the agmatine-treated human MC is not due to increased cell death. These results suggest that agmatine is a promising drug candidate for the treatment of human mesangial proliferative glomerulonephritis. ——— agmatine; polyamines; human mesangial cells; cell proliferation

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Agmatine is a metabolite of arginine via arginine decarboxylase (ADC). The kidney is unusual in displaying high constitutive ADC activity, and may be a principal contributor of systemic agmatine (Lortie et al. 1996). Agmatine has the capacity to regulate nitric oxide (NO) synthase activity (Galea et al. 1996; Abe et al. 2000; Satriano et al. 2001a) and has been ascribed roles in association with neurotransmitter receptors (Li et al. 1994), modulation of opioid analgesia (Kolesnikov et al. 1996), and as an ADP-ribose acceptor (Murayama et al. 1993). It is structurally analogous to polyamines (putrescine, spermidine and spermine) and can regulate intracellular polyamine content (Satriano et al. 1998).

Polyamines are required components for DNA replication, proliferation, and cell homeostasis (Pegg and McCann 1982; Tabor and Tabor 1982). Ornithine decarboxylase (ODC) is the first rate-limiting enzyme of polyamine biosynthesis. It is a proto-oncogene that is significantly elevated in proliferating cells and supplies polyamines required for cell cycle progression and growth. Increased polyamine uptake also accompanies growth as polyamine transport is stimulated by many of the same factors that induce ODC. Polyamines are autoregulated through induction of the inhibitory protein, namely (ODC-) antizyme, which inhibits both ODC for polyamine synthesis and cellular polyamine transport (Satriano et al. 1998). In previous studies we have shown that agmatine administration induces antizyme expression, depletes intracellular polyamine levels, and suppresses cellular proliferation in a transformed murine proximal tubule cell line (Satriano et al. 1998). Furthermore, agmatine utilizes, and is dependent upon, the polyamine transporter for cellular uptake (Satriano et al. 2001b).

The clinical classification of glomerulonephritis (GN) is based on pathogenesis, morphology or clinical presentation. A crucial event during the initial phase in many forms of GN is the proliferation of glomerular mesangial cells (MC). Increased ODC activity, and thus polyamine expression, plays an important role in the proliferation of cultured mesangial cells (Schulze-Lohoff et al. 1991). In anti-Thy-1 GN rat, an

experimental model of MC proliferative GN, agmatine administration suppresses ODC activity, polyamine levels, and MC proliferation. The anti-Thy-1 GN rats administered agmatine demonstrate decreased glomerular injury and extracellular matrix production and improved renal function relative to rats administered anti-Thy-1 antibody alone (Ishizuka et al. 2000).

In this context, we evaluated the antiproliferative effects of agmatine in human MC and the therapeutic potential of agmatine for human GN.

MATERIALS AND METHODS

Materials

Agmatine, putrescine, platelet-derived growth factor BB (PDGF-BB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

Human mesangial cells (CC-2559) from normal tissue were obtained from Cambrex Bio Science (Walkersville, MD, USA), were routinely grown as a monolayer in RPMI-1640 (Sigma) medium supplemented with 5% fetal bovine serum (FBS; Equitech-Bio Inc., Kerrville, TX, USA) containing 100 U Penicillin G potassium (Ban-yu Seiyaku, Tokyo) and 100 μ g/ml Streptomycin Sulfate (Meiji Seika, Tokyo). After two to three passages using trypsin/EDTA (Denka-Seiken, Tokyo), homogeneous cultures of human MC were obtained and used for all experiments.

Cell counting

In 35-mm dishes (Greiner bio-one, Frickenhausen, Germany), human MC were grown in RPMI-1640 supplemented with (1) 20% FBS or (2) 5% FBS + PDGF (20 ng/ml) or (3) 5% FBS, and in the absence or presence of 1 mM agmatine. Cell numbers were counted by hemocytometer each day.

4. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay

To examine the antiproliferative effects of agmatine, human MC on a 96-well flat-bottomed microplate (BD Biosciences, Franklin Lakes, NJ, USA) were grown for 5 days, and incubated with the MTT solution for 4 hours. The quantification of viable cells was assayed with the use of Cell Proliferation Kit I (Roche Molecular Biochemicals, Mannheim, Germany) and scanning multiwell spectrophotometer (MicroplateReader Model 550,

Bio-Rad Laboratories, Hercules, CA, USA). Absorbance of the formazan products were measured at 595 nm, with the reference wavelength of 655 nm.

Measurement of intracellular polyamine contents (HPLC: high-performance liquid chromatography)

Human MC were harvested in 100-mm dishes (Greiner bio-one) with each of the conditions as described above for 5 days. Cells were homogenized with a Teflon homogenizer, and polyamines (putrescine, spermidine and spermine) were extracted by the treatment with 0.3ml of 5% (w/v) trichloroacetic acid. Polyamine levels in the cellular extracts were measured by HPLC as previously reported (Igarashi et al. 1986).

Cellular DNA fragmentation enzyme-linked immunosorbent assay (ELISA)

The cellular DNA fragmentation ELISA is used to measure cell death by detection of BrdU (5-bromo-2'-deoxy-uridine)-labeled DNA fragmentation in the cytoplasm of affected cells (Roche Molecular Biochemicals). Human MC which were grown for 5 days in the absence or presence of 1 mM agmatine, the cells harvested and the BrdU-labeled DNA fragmentation in cytoplasmic lysates evaluated spectrophotometrically in the microplate reader described above.

Statistics analysis

All experimental data were expressed as the mean $\pm$ s.d. The data were evaluated with significance determined by Mann-Whitney's U-test. $P < 0.05$ was considered significant.

RESULTS

Agmatine inhibits proliferation of human MC

To determine if agmatine has antiproliferative effects on human kidney cells, we evaluated the effects of agmatine on human MC proliferation over a 5-day period (Fig. 1). Cellular proliferation of human MC supplemented with FBS (20% total) increased exponentially and was significantly higher than that of MC in the control media containing 5% FBS. The antiproliferative effects of agmatine on human MC were significant from day 3. Agmatine suppressed growth by 48.5% and 62.5% for 5% and 20% FBS, respectively.

Polyamine transport is correlated with the

rate of proliferation and growth. As agmatine utilizes the polyamine transporter, we compared the sensitivity of the human MC at normal (5% FBS) and stimulated growth rates (20% FBS and 5% FBS + PDGF) to varying concentrations of agmatine (Fig. 2). Our data from the MTT proliferation assay show that the antiproliferative effects of agmatine increased significantly in both conditions of enhanced growth, i.e., 20% FBS and 5% FBS + PDGF (5% FBS vs. 20% FBS, 5% FBS vs. 5% FBS + PDGF: $p < 0.05$). The antiproliferative effect peaked at 1 mM agmatine in the stimulated cells. The suppressive effects of agmatine on proliferation of human MC in this study were: 20% FBS > 5% FBS + PDGF >> 5% FBS.

Effects of agmatine on polyamine metabolism

To determine whether agmatine promotes the antiproliferative effects on human MC by altering their polyamine concentrations, putrescine, spermidine and spermine contents were measured by HPLC. One mM agmatine reduced total polyamine content, most remarkably of putrescine and spermidine (Fig. 3).

Effects of polyamine supplementation

To confirm that the antiproliferative effects observed with agmatine are due to polyamine depletion, human MC were incubated in 20% FBS for 5 days with various concentrations of agmatine in the absence or presence of 1 mM putrescine. Cell viability was evaluated by MTT proliferation assay. Putrescine supplementation eradicated the antiproliferative effects of agmatine on mitogenic human MC (Fig. 4).

Cell death analysis

The decrease in cell numbers we observe with agmatine administration in the MTT proliferation assay may be due to suppression of cell division and/or to an increase in cell death. Cell death was evaluated by the cellular DNA fragmentation assay. In untreated cells the absorbance of BrdU labeled DNA fragmentation was 0.07 ± 0.02 . This was essentially unchanged with agmatine administration where the absorbance was 0.06 ± 0.01 . Moreover, cell death also did not appear

Fig. 1. Time course effect of agmatine.

Cells were cultured in medium containing (A) 5% FBS or (B) 20% FBS and counted by hemocytometer. * $P < 0.05$ when compared with agmatine untreated cells.

Fig. 2. Dose dependent effect of agmatine.

(A) % of control (untreated) cells, and (B) absorbance of cells in the presence of varying concentrations of agmatine as indicated. Cell viability was evaluated by the MTT proliferation assay. Each data point represents the mean ($\pm$ S.D.) for three experiments. * $P < 0.05$ when compared with 5% FBS treated cells.

A **B** **C**

Fig. 3. Intracellular polyamine (A; putrescine, B; spermidine, C; spermine) concentrations. Lanes: (1) 5% FBS; (2) 5% FBS + 1 mM agmatine; (3) 20% FBS; (4) 20% FBS + 1 mM agmatine; (5) 5% FBS + PDGF (20 ng/ml); (6) 5% FBS + PDGF (20 ng/ml) + 1 mM agmatine. Data presented are the mean value for 2 times measurements.

Fig. 4. Antiproliferative effect of agmatine is suppressed by polyamine supplementation. Cells were in the presence of varying concentrations of agmatine as indicated, cultured in medium containing 20% FBS. Cell viability was evaluated by MTT proliferation assay. Each data point represents the mean ($\pm$ S.D.) for three experiments. * $P < 0.05$ when compared with putrescine untreated cells.

to be a factor as obvious morphological characteristic such as extensive swelling of the cell, distension of various cellular organelles, and cell lysis due to loss of membrane integrity in light-microscopic examinations was not found on human MC in the presence of 1 mM agmatine (data not shown). Thus, cell death does not appear to be a contributing factor to the decreased cell numbers observed by MTT in human MC cells administered agmatine.

DISCUSSION

MC proliferation is a pathological characteristic of glomerular diseases including IgA nephropathy, membranoproliferative glomerulonephritis, lupus nephritis, and diabetic nephropathy. Regardless of the underlying injurious mechanism, altered control of MC proliferation appears to play an important role in the pathogenesis of progressive glomerular abnormalities leading to glomerulosclerosis. Therefore, preventing MC hypercellularity can have beneficial downstream effects on decreased renal function. Although corticosteroid, immunosuppressive, and antihypertensive agents are inhibitors of MC proliferation, specific antiproliferative agents in these human diseases has not been established thus far, a fact that may be due to the lack of orally applicable compounds without severe side effects.

The present study is the first to examine the antiproliferative effects of agmatine on human MC. Since agmatine is an endogenous molecule, it may be expected to show a low risk of adverse effects. Previous experiments have shown that the antiproliferative effects of agmatine are due, in large part, to modulation of polyamine metabolism (Satriano et al. 1998; Gardini et al. 2003; Molderings et al. 2004). In rat liver hepatoma cells, the antiproliferative effects of agmatine are concentration dependent. In addition to its effects on cellular polyamines, agmatine can regulate NO synthase activity, specifically iNOS activity (Galea et al. 1996; Abe et al. 2000; Satriano et al. 2001a). iNOS mediated NO generation plays a significant role in the early phase of GN. Importantly, agmatine is purported as a gating mechanism between these two arginine dependent pathways in GN, i.e.,

the early NO anti-proliferative/bactericidal phase and the later polyamine pro-proliferative, repair phase (Satriano 2004). In vivo studies using the anti-Thy-1 GN rat model support the proposed positive effects of agmatine in GN, in particular by suppressing proliferation and improving of renal function (Ishizuka et al. 2000). Thus, in the current study, we evaluate the antiproliferative effects of agmatine in proliferating human MC to detect the therapeutic potential of agmatine for human GN.

Polyamine transport correlates with cell cycle progression and proliferation. Agmatine is imported by the polyamine transport system in mammalian cells (Satriano et al. 2001b), suggesting that cells with increased proliferative kinetics would be more sensitive to its effects. Our results in cell counting and MTT proliferation assay show that proliferating human MC stimulated with mitogenic agents are indeed significantly more sensitive to the antiproliferative effects of agmatine than unstimulated cells. Agmatine administration also reduces total intracellular polyamine content, most notably of putrescine and spermidine. The marked declines of putrescine and spermidine, but not spermine, are in agreement with the agmatine results in rat liver hepatoma cell line (Gardini et al. 2003) and MCT cells (Satriano et al. 1998), and with selective inhibition of ODC activity with difluoromethylornithine (DFMO) in a murine B cell line (Nitta et al. 2002). Administration of putrescine completely eradicates the antiproliferative effects of agmatine (Fig. 4), suggesting that putrescine competes with agmatine for the same transport system then reducing agmatine uptake by human MC. It is suggested that agmatine suppresses cellular proliferation in human MC by attenuation of intracellular polyamine levels.

The results of cell death analysis suggest that suppression of proliferation in human MC is not due to increase of cell death, but to a change in the mitotic rate. Increasing concentrations of agmatine markedly shifted the cell cycle population into G1 phase arrest in a transformed murine fibroblast cell line, relative to the non-transformed parental line (Isome et al. 2002).

In this study, human MC are shown to be sensitive to the antiproliferative effects of agmatine. The relation between these effects and the decrease of intracellular polyamine content are in agreement with previous reports on many other cell lines (Satriano et al. 2001b; Gardini et al. 2003; Molderings et al. 2004). Furthermore, human MC stimulated to increase their proliferative kinetics are significantly more sensitive to the antiproliferative effects of agmatine than normally cultured cells. Thus, agmatine would preferentially be selectively imported into, and affect, proliferating cells. These results suggest that agmatine is a promising drug candidate for the treatment of human proliferative GN.

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