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EFFECT OF MITOMYCIN C ON PROSTACYCLIN SYNTHESIS BY HUMAN ENDOTHELIAL CELLS

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Abstract—The effect of mitomycin C (MMC) on the biosynthesis of prostacyclin was tested in culture of human umbilical cord vein endothelial cells. A 30% inhibition of the thrombin-stimulated prostacyclin synthesis by MMC was observed at concentrations of the same order as those found in MMC-treated patients (3 μg/ml as compared with the peak plasma concentration varying between 0.4 and 3.2 μg/ml (J. Den Hartigh *et al.*, *Cancer Res* **40**: 5017–5021, 1983)). This inhibition was found for incubation times ranging from 15 to 30 min during which the cell viability was unaltered. Under these conditions it was found that the release of von Willebrand factor by the endothelial cell was unaffected. Since MMC toxicity in man is expressed by a chronic haemolytic and uraemic syndrome, the inhibitory capacity of MMC on prostacyclin synthesis favours the hypothesis that a deficiency in prostacyclin synthesis leads to the development of this syndrome in man.

Mitomycin C (MMC) is a cytotoxic antibiotic with antitumoral activity, widely used essentially in the treatment of intestinal and breast cancer [1]. Its nephrotoxic effect has been known for some time [2], but recently MMC has been implicated in haemolytic–uraemic syndrome (HUS) ([3, 4] reviewed in Ref. 30). Clinically, HUS is characterized by haemolytic anaemia, schizocytosis and thrombopenia associated with a glomerulopathy. These clinical features are also characteristic of thrombotic thrombocytopenic purpura (TTP). However, TTP and HUS are clinically distinguishable as the former shows a prevalence of neurological symptoms, while the latter is associated with proteinuria and renal failure. The pathogenesis of TTP and HUS still remains uncertain. The occlusive microthrombi found in the small arterioles and capillaries are primarily composed of platelets but may also contain fibrin and leukocytes [5, 6]. It has been suggested that these microthrombi are the result of disseminated intravascular platelet consumption or platelet aggregates at sites of discontinuous vascular endothelial cell injury [7]. Remuzzi *et al.* have proposed that both TTP and HUS becomes manifest in patients deficient in a plasma factor normally stimulating vascular prostacyclin activity [8]. Thus TTP and HUS may represent a local relative prostacyclin deficiency and the maintenance of a homeostatic balance [9] between thromboxane A2 (formed by aggregating platelets) and prostacyclin (produced by the vessel wall) may well be disturbed in both diseases. This report presents the results of experiments investigating the inhibitory effects of MMC on the thrombin stimulated biosynthesis of prostacyclin in cultures of human endothelial cells.

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MATERIALS AND METHODS

Endothelial cell isolation and identification. Human umbilical vein endothelial cells were obtained by the method of Jaffe [10] with some minor modifications. Briefly, umbilical cords were collected within 48 hr of delivery in a sterile saline solution (Hepes buffered saline: HBS; 0.14 M NaCl, 4 mM KCl, 11 mM glucose, 15 mM Hepes) containing antibiotics. The vein was filled with 10 ml 0.1% collagenase solution, placed in a sterile beaker containing HBS and incubated at 37° for 10 min. After incubation, the vein was perfused with 20 ml HBS and the cell containing solution collected in a sterile 50 ml tube and centrifuged at 100 *g* for 10 min at 20°. The supernatant fluid was discarded, the cell pellet resuspended in culture medium (3 ml/cord) and the cells were plated on fibronectin coated dishes (10 μg/cm^2). Normally, three 35 mm culture dishes were seeded from a single cord. Cells were routinely grown in M199/RPMI 1640 (v/v) medium supplemented with 30% heat-inactivated human serum, 1 mM L-glutamine, 15 mM hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, in a humidified atmosphere, 95% air, 5% CO_2. The medium was changed twice weekly. Under these conditions, the cells reached confluence in 5–6 days. A typical monolayer of polygonal shaped cells was observed at confluence by phase microscopy. The presence of von Willebrand factor was demonstrated by indirect immunofluorescence using a rabbit antiserum [10]. The following procedure was applied for the mitomycin C assay. Cells were washed three times with Hank's balanced salt solution (HBSS) and 0.9 ml HBSS (without phenol red or antibiotics) was added. MMC in 0.1 ml HBSS (0.1 ml HBSS for controls) was then added. Triplicate incubations were carried out at 37° in a humidified atmosphere 95% air, 5% CO_2.

6-Keto-PGF1α radioimmunoassay. The incubation medium obtained as above was extracted using a 1:1 mixture of cyclohexane:ethyl acetate after adjusting to pH 2.0 with citric acid. The extract was separated by chromatography using a silicic acid column. Fractions corresponding to 6-keto-PGF1α were collected and measured for their PG content using a 6-keto-PGF1α antiserum. The procedure followed was exactly that described by Dray *et al.* [12], using a charcoal (1%) dextran T 70 (0.1%) suspension for the separation of the bound and unbound forms of ligand. With this procedure, a blank value ranging between 150 and 200 pg was obtained when 1 ml plain HBSS was extracted and assayed.

Fibronectin was isolated from human plasma using a gelatin-Sepharose affinity chromatographic procedure [13].

Human factor von Willebrand was measured by a radioimmunoassay using a monoclonal antibody (Immunotech, France).

Culture media M199 and RPMI 1640, hepes, antibiotics and trypsin were obtained from Boeringher, France. Hank's balanced salt solution was from Gibco. The calcium ionophore A23187 was from Lilly Research Laboratory. Thrombin (human, 3000 units/mg of protein), 6-keto-PGF1α and standard laboratory chemicals were purchased from Sigma. Collagenase type I CLS was from Worthington. [^{3}H]-6-keto-PGF1α was from the Radiochemical Centre, Amersham. Antiserum for the 6-keto-PGF1σ radioimmunoassay was obtained from the Institut Pasteur, Paris. Silicic acid (100 mesh) was from Mallinckroodt. Rabbit polyclonal antibody against human factor von Willebrand was the generous gift of Dr. D. Meyer, Paris. Human blood serum was prepared from a pool of 50–100 blood donors. The blood was allowed to clot 24 hr at 4°. The serum was then heat inactivated (30 min; 58°), sterilized by filtration through 0.2 μm filters and stored at −20° until use.

Statistical analysis. Statistical significance was determined by Student's *t*-test. A P value < 0.01 was selected to denote statistical significance between groups.

Table 1. Effect of thrombin, ionophore A23187, and acetylsalicylic acid on prostacyclin synthesis

Stimulus	6-Keto-PGF$_{1\alpha}$ (pg/10 min/10^6 cells)	
	−	+ASA
—	350 ± 41	0
Thrombin 1 U/ml	3625 ± 425	127 ± 31
Thrombin 5 U/ml	4432 ± 520	153 ± 46
Ionophore A 23187 10 μM	6048 ± 840	180 ± 38

Endothelial cells were incubated for 30 min with or without 100 mM acetylsalicylic acid (ASA) in serum-free medium and subsequently stimulated with thrombin or A23187. After 10 min the amount of 6-keto-PGF1α was measured. Mean values and standard deviations were calculated from three experiments.

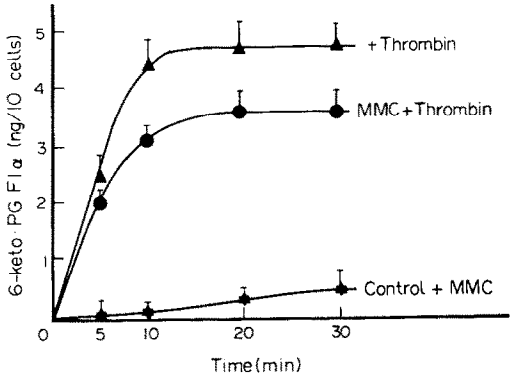

Fig. 1. Time course of the effect of thrombin and mitomycin C on prostacyclin synthesis by endothelial cells. Cells were preincubated for 15 min with or without 3 μg/ml mitomycin C in serum-free medium and subsequently stimulated with thrombin (5 U/ml). Results are expressed as the mean ± SEM of three separate incubations.

RESULTS

The production of 6-keto-PGF1α by endothelial cells was considerably variable in preliminary experiments. To minimize these variations, cells were always used at confluence and one day following change of medium. Under these conditions, human umbilical cord vein endothelial cells generated only small amounts of 6-keto-PGF1α, in the absence of stimulation (Table 1). The quantity of 6-keto-PGF1α produced by these cells was greatly enhanced by incubation with thrombin or ionophore A23187 (Table 1). However, basal and stimulated biosynthesis was completely inhibited by acetyl salicylic acid (Table 1).

To study the effects of MMC on PG biosynthesis, endothelial cells were preincubated at 37° for 15 min with or without MMC. The medium was removed and replaced with fresh medium containing the same MMC concentrations and 0 or 5 U thrombin/ml. Culture dishes were then further incubated at 37°. As shown in Fig. 1, MMC had no effect on the 6-keto-PGF1α biosynthesis of unstimulated cells. However, when endothelial cell monolayers were preincubated with 3 μg MMC/ml, the release of 6-keto-PGF1α in the presence of 5U thrombin/ml was inhibited by 30% (Fig. 1). The effect was dose dependent as shown in Table 2. Maximum inhibition (37%) occurred at 10 μg MMC/ml. Further decrease in 6-keto-PGF1α biosynthesis was not observed at higher MMC concentrations (data not shown). To exclude the possibility that the inhibitory effect observed was due to non release of 6-keto-PGF1α from the cells rather than an inhibition of synthesis, PG extraction experiments were performed on the cells and the medium. The same inhibitory effect of MMC was observed (data not shown). Calcium ionophore induced production of 6-keto-PGF1α was similarly inhibited (Table 2).

As shown in Fig. 2, thrombin at a concentration

Table 2. Effect of mitomycin C on prostacyclin synthesis

Stimulus	Mitomycin C concentration (μg/ml)				
	0	1	3	5	10
—	360 ± 32	370 ± 53	270 ± 41	322 ± 45	300 ± 45
Thrombin 5 U/ml	4523 ± 437	3985 ± 452	3204 ± 200*	2920 ± 190*	2813 ± 185*
Ionophore A 23187 10 μM	6080 ± 516	5910 ± 503	4161 ± 431*	3880 ± 288*	3612 ± 264*

Endothelial cells were incubated for 15 min with mitomycin C in serum-free medium and subsequently stimulated with thrombin (5U/ml) or ionophore A23187 (10 μM). After 10 min the amount of 6-keto-PGF1α was measured. Results (pg/10 min/106 cells) are expressed as mean ± SEM of 3 separate incubations.

* $P < 0.01$ vs the values obtained with the stimulus alone.

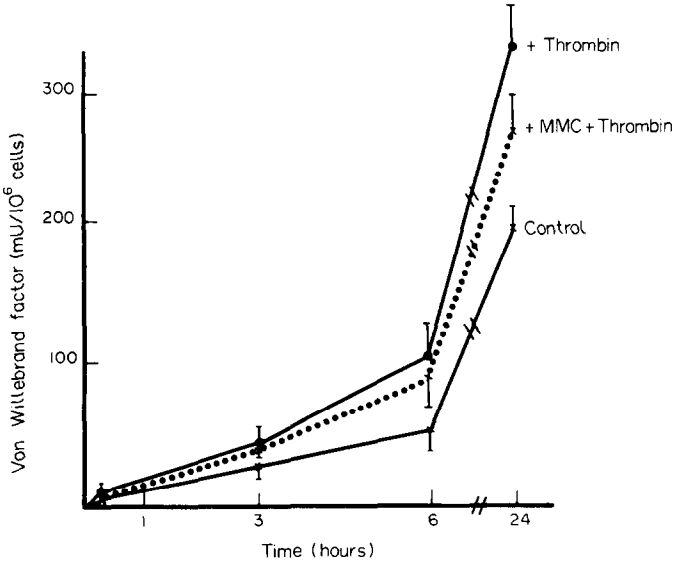

Fig. 2. Time course of the effect of thrombin and mitomycin C on the release of von Willebrand factor. Cells were preincubated for 15 min with or without 3 μg/ml mitomycin C in serum-free medium and subsequently stimulated with thrombin (5 U/ml). Each value represents the mean ± SEM calculated from triplicate wells of cells in a representative experiment.

of 5 U/ml, stimulated the release of von Willebrand factor from endothelial cells. This release was slightly inhibited by MMC at 3 μg/ml. This inhibitory effect was not significantly different from controls for incubations inferior to 6 hr. After this period a 20% inhibition of the thrombin-stimulated release of von Willebrand factor by MMC was detectable.

Since MMC can induce cell death at the concentrations used, cell survival after MMC treatment of endothelial cell monolayers was measured. The cells were exposed to serum-free medium, containing 1% human serum albumin and increasing concentrations of MMC for 30 min to 24 hr. The medium was then discarded and the cells washed twice with HBSS, harvested by trypsinisation (0.25% trypsin) and counted. Cells (10^5) were then seeded in 1 ml culture medium in 35 mm dishes. Controls consisted of cells treated in exactly the same way but without MMC. Cell viability was defined as their capacity to give rise to a cell monolayer. These experiments showed that low MMC concentrations (1–3 μg/ml) and short exposure times (less than 1 hr) had no effect on cell viability. For higher doses of MMC or longer incubations, the time needed for cells to reach confluence increased to up to 10 days (5 days for control cultures). Cells exposed to 3 μg/ml MMC for 24 hr never reached confluence (data not shown).

DISCUSSION

The results reported above demonstrate that MMC inhibits the thrombin induced synthesis of prostacyclin by human endothelial cells in culture. This inhibition occurred at MMC concentrations of the same order as those found in the plasma of MMC treated patients (3 μg/ml as compared with the peak

plasma concentration varying between 0.4 and 3.2 μg/ml [14]). The endothelial cell viability was unaffected at these MMC concentrations, demonstrating that the inhibitory effect observed with MMC was not a consequence of cell death.

At the concentrations acting on prostacyclin biosynthesis, MMC had no inhibitory effect on the thrombin induced release of von Willebrand factor, excepting for long exposure times where cell viability was affected. This indicates that the inhibitory effect of MMC at therapeutic concentrations appears more specific for prostacyclin synthesis.

Prostacyclin is the most powerful inhibitor of platelet aggregation known [15]. It is mainly synthesised by the vascular endothelial cells [16], and its inhibitory properties oppose the pro-aggregating activity of thromboxane A2, which is secreted by the platelets [17]. An imbalance in the synthesis of either prostacyclin or thromboxane A2 would result in an anomaly in platelet aggregation [9].

A deficiency in prostacyclin synthesis has been suggested as the factor inducing HUS. Several authors have reported finding very low levels of plasmatic prostacyclin in both HUS and PTT [8, 18, 19–21]. These low levels of circulating prostacyclin have been explained by a very rapid plasmatic degradation of prostacyclin [18], a deficiency in a plasmatic factor which stimulates prostacyclin synthesis [19] or the action of a plasmatic inhibitor [8, 22].

MMC is an alkylating agent. Its primary cellular effect is the inhibition of DNA synthesis [23], especially when the cells are exposed to MCC during the late G1 and early S-phase of the cell cycle [24, 25]. MMC binding to DNA takes place both *in vivo* and *in vitro* and produces DNA cross-links. Few biochemical studies to date have been undertaken on the effects of MMC on cell metabolism, and those found in the literature deal mainly with the toxic effects of MMC on cell growth and viability [26, 27]. However, the inhibitory effects of MMC on cyclic AMP phosphodiesterase [28] and testicular steroidogenesis [29] have been reported.

MMC is frequently used in the treatment of different types of cancer. The first secondary effect resulting from MMC treatment is myelosuppression. However, MMC may also provoke pulmonary and renal disorders [1, 30]. MMC is often used in combination with other antitumor agents, e.g. 5-fluorouracyl, but several authors have demonstrated the involvement of MMC in the onset of HUS [31, 32]. MMC-induced HUS is unusual in that it appears progressively several weeks after treatment has begun, and that glomerular mesangiolysis is near constant on renal biopsies in addition to clear, fibrin like subendothelial deposits [30]. MMC is the first drug known to provoke HUS. MMC's capacity to inhibit prostacyclin synthesis in endothelial cells favours the hypothesis that a deficient prostacyclin synthesis plays an important role in the development of HUS.

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