Toxic effects of methylmethacrylate monomer on leukocytes and endothelial cells in vitro

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The influence of methylmethacrylate monomer (MMA) on the cellular integrity of monocytes, granulocytes and endothelial cells in vitro was investigated. Clinically relevant blood concentrations of MMA (i.e., 5–10 µg/mL) were clearly cytotoxic to all cell types studied, as evidenced by the release of lactic dehydrogenase (LD) and ^{51}Cr, and increased uptake of trypan blue (vital staining). Scanning electron microscopic examination of cells treated with 10 µg/mL MMA showed marked signs of cytotoxicity after 1 min incubation, and after 30 min the majority of the cells were totally disintegrated. These findings may have clinical bearing on intraoperative cardiorespiratory dysfunction and deep vein thrombosis in MMA-fixed joint replacement surgery.

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It has long been known that the liquid monomer component of methylmethacrylate (MMA) is cytotoxic (Deichmann 1941). Implantation of acrylic cement in human beings has repeatedly been reported to be associated with fatal cardiorespiratory collapse (Rinecker 1980, Ferris and Kinsella 1984, Dahl et al. 1988a, Duncan 1989).

There appears to be a higher incidence of deep vein thrombosis (DVT) and, in particular, proximal DVT after cemented hip and knee joint replacements, compared to non-cemented total joint replacements (Stamatakis et al. 1977, Lynch et al. 1988, Francis et al. 1989, Høgevold et al. 1990). Recently we have demonstrated that MMA in doses equivalent to blood concentrations found in patients during total hip replacement (THR) (Svartling et al. 1985, Wenda et al. 1988, Dahl et al. 1992a) modulate blood coagulation triggering in different ways (Dahl et al. 1992b).

The pathophysiological aberrations leading to the development of peroperative CRC and postoperative proximal DVT in patients undergoing cemented THR, seem to include both activation of coagulation (Dahl et al. 1988b, 1992) and a possible harmful effect of MMA which may cause damage to blood cells and endothelial cells (Dahl et al. 1992b). In order to substantiate this hypothesis, we have determined the threshold concentrations of MMA necessary to cause damage to human leukocytes and endothelial cells in vitro.

Material and methods

Endothelial cells were prepared from umbilical cord veins essentially as described by Jaffe et al. (1973). Umbilical cord veins were cannulated and flushed with phosphate-buffered saline (PBS) of pH 7.4 to remove blood and then filled with 0.1% collagenase solution (Type I, Sigma Chemical Company, St. Louis, MO, U.S.A.) for 15–20 min at 37 °C. Detached cells were recovered by flushing with PBS and collected by centrifugation at 500 g for 7 min. The cells were resuspended in RPMI 1640 cell culture medium (Gibco, Paisley, Scotland) containing 20 percent heat-inactivated (56 °C, 30 min) fetal calf serum (FCS) (Bio-Whittaker, Walkersville, MD, U.S.A.), penicillin 50 U/mL, streptomycin 50 µg/mL, fungizone 2.5 µg/mL and distributed in 24 well-tissue culture plates (Costar, Cambridge, MA, U.S.A.) each well containing 1 mL and incubated at 37 °C in humidified air with 5 percent CO_2. After 24 hours the medium was removed, the cells washed gently 3 times with PBS to remove non-adherent cells, before fresh medium (as described) was added. Primary cell cultures were used when confluence occurred usually after 3–4 days. The cultured cells were identified as endothelial cells by their typical cobblestone appearance, and selected dishes were also periodically studied with indirect immunofluorescent staining for vWF antigen.

Monocytes were prepared from buffy coats obtained from healthy blood donors. After sedimentation of erythrocytes by adding 1 percent dextran-70 (Macrodex, Kabi Pharmacia, Stockholm, Sweden), the

supernatant was pipetted off, layered on Lymphoprep (Nycomed, Oslo, Norway) and centrifuged for 30 min at 400 g. The mononuclear cell fractions were harvested, and washed three times in 20 mL PBS, pH 7.4. The cells were then resuspended in RPMI 1640/20 percent FCS medium and seeded (10^7 mononuclear cells/well) in 24 well-tissue culture plates and monocytes left to adhere for 2 hours at 37 °C. Non-adherent cells (mainly lymphocytes) were removed by three washes with PBS, and the monocyte cultures were regularly more than 90 percent pure, judged by morphology after staining with a modified May-Grünwald-Giemsa staining method (Diff-Quick, Merz and Dade, Harleco, Philadelphia, PA, U.S.A.).

Granulocytes were prepared after resuspending the bottom pellet (containing granulocytes and erythrocytes) of the Lymphoprep gradient centrifugation in PBS and layering it on 80 percent Percoll (Pharmacia, Uppsala, Sweden) before centrifugation at 400 g for 30 min. The fractionated cells (>97 percent granulocytes, as judged by morphology) were washed twice in PBS before resuspension in RPMI 1640/20 percent FCS and seeding in tissue culture clusters (2×10^6 cells mL).

Cytotoxicity assays

Lactate dehydrogenase release. As a measure of cell damage, the cytosol enzyme lactate dehydrogenase (LD) was measured in lysates and supernatants of endothelial cells, monocytes and granulocytes after incubation with MMA (Palacos, Schering Corp, Kenilworth, NJ, U.S.A.) in various concentrations for different lengths of time. Selected cultures were also treated with thrombin (10 U/mL) (USP Thrombostat, Parke-Davis, Morris Plains, NJ, U.S.A.) in combination with MMA. LD activity was measured according to Decker et al. (1988). Media control levels for each time-point were subtracted from the values for supernatant LD activity. The percentage of LD release was calculated as follows: percent lysis = experimental LD release ÷ spontaneous LD release divided by maximum LD release ÷ spontaneous LD release × 100.

^{51}Cr-release cytotoxicity assay. ^{51}Cr was used for quantitative studies of MMA-induced lysis of cells. ^{51}Cr-labeled Na_2CrO_4 is incorporated into living cells and reduced to the trivalent form upon attachment to proteins and other cell constituents. When a cell is lysed, ^{51}Cr-labeled proteins leak out. As the trivalent ^{51}Cr cannot be reutilized by other cells, measurement of ^{51}Cr radioactivity in the medium gives a measure of cell lysis (Wigzell 1965). Monocytes, granulocytes (2×10^6 cells per mL cell culture medium) or confluent endothelial cells were labeled with 20 μCi of sodium (^{51}Cr) chromate (specific activity 6.6 GBq/mg Cr, Institutt for Energiteknikk, Kjeller, Norway) for 16 hours at 37 °C, washed 3 times in RPMI 1640/20 percent FCS medium before addition of MMA at varying concentrations. Incubations were continued for 16–20 hours and the radioactivity of the media (counts per minute = CPM) was counted in a gamma counter (Packard, Chicago, U.S.A.). The degree of cell lysis was calculated as follows: percent lysis = CPM (experimental ÷ spontaneous release)/CPM (maximum ÷ spontaneous release) × 100.

Trypan blue cytotoxicity assay. At the end of the incubation period and after the cells had been washed according to the standard washing procedure, the remaining cells were incubated with 0.2 percent trypan blue dissolved in 0.15 M NaCl. After 5 min incubation at room temperature, the proportion of stained (dead) cells was calculated by cell-counting in a Bürker hemocytometer.

Scanning electron microscopy. Endothelial cell cultures or cytospin preparations of granulocytes or mononuclear cells that had been treated with MMA were fixed in 2 percent glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), dehydrated in graded ethanols and critical-point dried (Polaron E 3100 Critical Point Drier, Polaron Equipment Ltd., Watford, England), using carbon dioxide as the transitional fluid. The preparations were then adhered to carbon stubs and coated with a 300 Å thick layer of gold/palladium alloy in a Polaron E 5100 Sputter Coater. The specimens were examined and photographed with a Philips SEM 515 (Eindhoven, Holland) microscope, using a conventional secondary detector, at magnifications ranging from × 163 to × 10900.

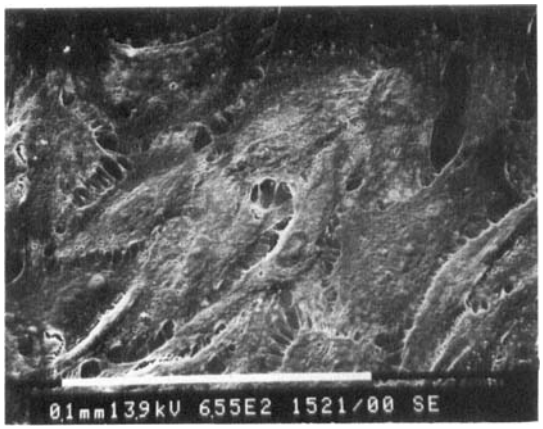
Results

Scanning electron microscopy

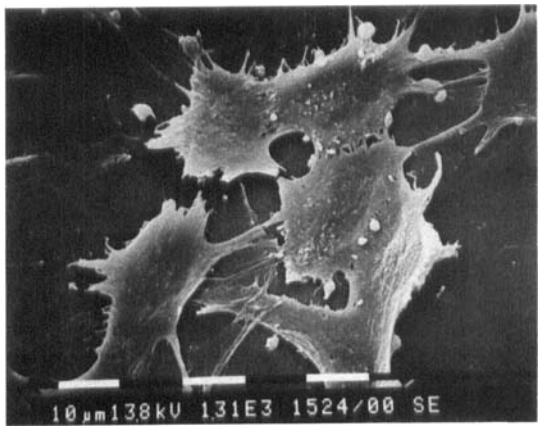
Scanning electron micrographs of control endothelial cells showed smooth-surfaced endothelial cells with distinct intercellular boundaries, slightly raised cell nuclei and a few plasma membrane microvilli. Within minutes after the addition of MMA (10 μg/mL), the endothelial cells lost their normal polygonal shape and spread-out appearance. The cells lost their adherence to one-another and to the substratum, rounded up and were eventually shed from the surface, leaving only remnants of the basal cell membrane behind (Figure 1).

Exposure of monocytes to MMA (10 μg/mL) resulted in immediate cell enlargement and loss of membrane folding.

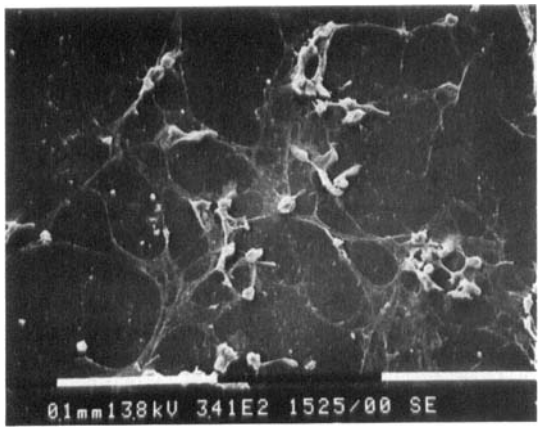
Figure 1. Scanning electron micrographs of endothelial cell cultures exposed to MMA (10 µg/mL).



Normal, near-confluent endothelial cells, ×655.



Cells exposed to MMA for 1 min showing cell retraction and detachment, ×1310.



30 min later showing cellular rounding-up and extensive cell loss, leaving remnants of basal cell membranes on the substratum, ×341.

This was followed by shedding of membrane vesicles or fragments, membrane disintegration and total cellular disorganization affecting the majority of cells after 30-min incubation (Figure 2).

Granulocytes exposed to MMA (10 µg/mL) showed immediate retraction of pseudo-/filopodia that had evolved as a result of glass adherence and caused the formation of large membrane blebs. The numerous delicate membrane protrusions of normal granulocytes disappeared rapidly (1 min exposure) as the cell became distended and acquired a smoother surface. Membrane dissolution and exposure of cytoplasmic constituents evolved, and after 30 min exposure, most of the cells were totally destroyed (Figure 3).

Cytotoxicity assays (Table 1)

Slightly increased LD concentrations were found in cellconditioned media containing 5 µg/mL MMA, irrespective of cellular origin, i.e., monocytes, granulocytes or endothelial cells. A near half-maximal LD release was obtained with MMA concentrations 10 µg/mL and maximal release was found at about 40 µg/mL for both granulocytes, monocytes and endothelial cells.

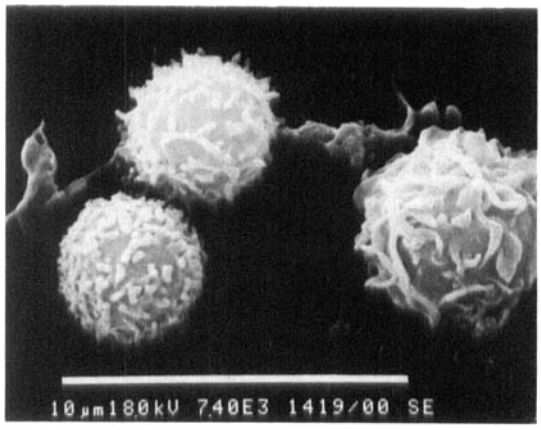
Costimulation of granulocytes (and endothelial cells, data not shown) with various concentrations of MMA and a fixed amount of thrombin (10 U/mL) (thrombin is generated in huge amounts in the plasma of patients undergoing total hip replacement operations) (Dahl et al. 1988b, 1993) did not shift the dose-response curves for MMA as regards LD release; 10 µg/mL MMA was still the critical cytotoxic dose.

The cytotoxic effect of MMA was also assessed by release of ^{51}Cr from prelabeled cells of the three categories and by trypan blue vital staining. Dose-response relationships obtained by these cytotoxicity tests (Wigzell 1965, Decker et al. 1988), very closely paralleled the LD release.

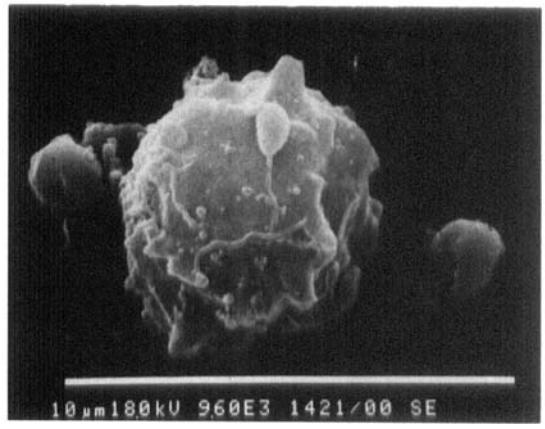
Discussion

Acrylic cement is frequently used in reconstructive orthopedic surgery and has been associated with perioperative cardorespiratory dysfunction and postoperative deep vein thrombosis, in particular at the site of cemented areas (Stamatakis et al. 1977, Rinecker 1980, Ferris and Kinsella 1984, Dahl et al. 1988a, Lynch et al. 1988, Duncan 1989, Francis et al. 1989, Høgevold et al. 1990). A substantial intraoperative activation of the coagulation system occurs during bone traumatization with a second wave of increased coagulation at the end of the first postoperative week

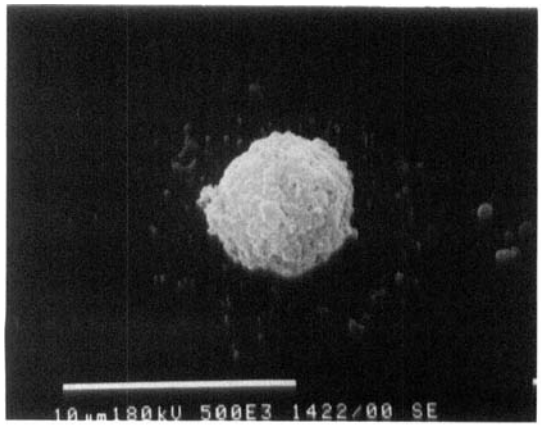
Figure 2. Scanning electron micrographs of mononuclear blood cells (monocytes and lymphocytes) exposed to MMA 10 μg/mL.



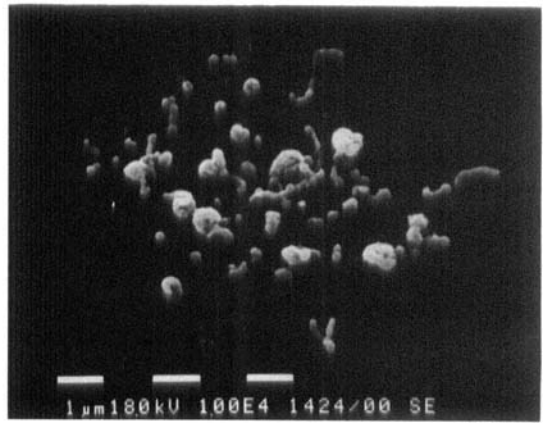
Normal cells (2 lymphocytes and 1 monocyte), ×7400.



After MMA exposure for 1 min showing monocytic cell distension and loss of membrane folding, ×9600. Note also the distended appearance of two blood platelets, compared to the platelets seen in A.



10 min exposure showing beginning membrane dissolution, ×5000.



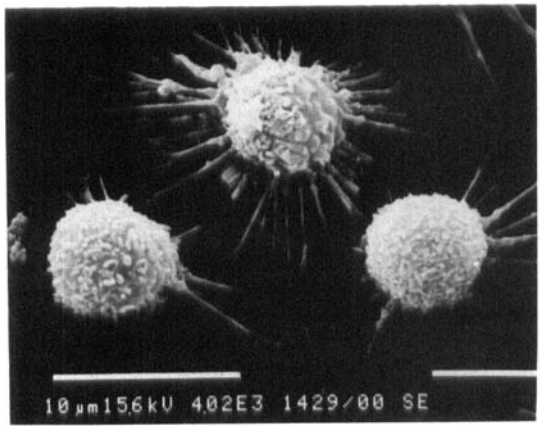
30 min exposure showing total cellular disintegration, ×10000.

Table 1. Cytotoxicity (percent lysis) of varying concentrations of MMA. Incubation time 16 hours. Values given are the mean of 6 cultures

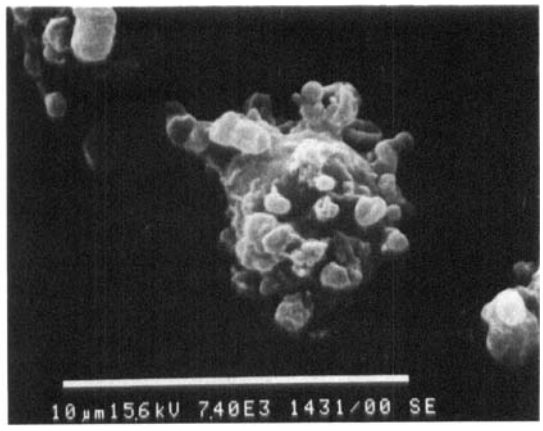
Cell	Release method	MMA concentration (μg/mL)						
		0.01	0.1	1	5	10	25	40
Monocytes	LD	10	9	12	25	51	67	81
	^{51}Cr	3	3	5	12	57	79	92
Granulocytes	LD	0	0	0	2	81	93	94
	^{51}Cr	8	4	3	9	19	62	85
Endothelial cells	LD	1	1	1	19	52	74	88
	^{51}Cr	0	0	0	4	36	78	97

following THR surgery (Dahl et al. 1988b, 1992a, 1993). Introduction of MMA following the bone traumatization, may add potentially harmful effects on blood cells and endothelial cells, specially of collecting veins draining the cement-impacted area. Activation of coagulation and the additional cytotoxic effect of MMA may be of importance both for the intraoperative cardiorespiratory and the postoperative vascular events, although precipitated at different phases of the per- and postoperative periods.

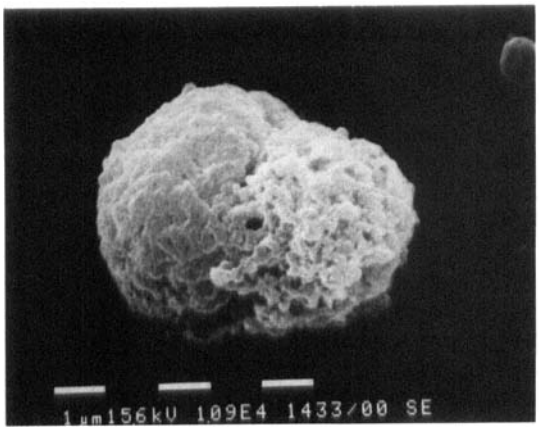
Figure 3. Scanning electron micrographs of granulocytes exposed to MMA 10 µg/mL.



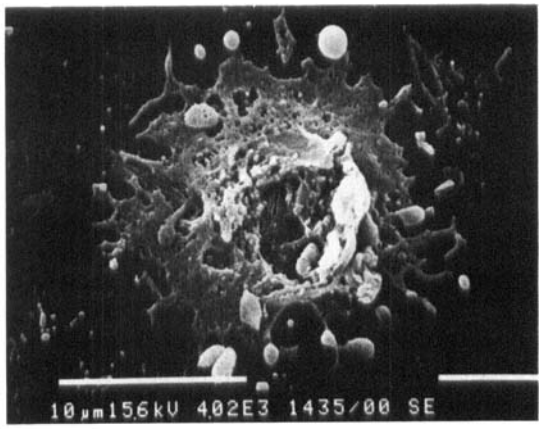
Normal cells with filopodia due to glass spreading, ×4020.



After exposure to MMA for 1 min showing retraction of filopodia and loss of membrane foldings, ×7400.



10 min exposure showing membrane disruption and expulsion of cytoplasm, ×10900.



30 min exposure showing complete cellular disintegration with plasma membrane remnants in the cytoplasmic mass, ×4020.

MMA is a pharmacologically active chemical substance which has been shown to have local and systemic effects in several experimental studies (Deichmann 1941, Bright et al. 1972, Homsy et al 1972, Ohnsorge and Kutzner 1974, Linder 1976, Wong et al. 1977). During cemented THR, microembolism, mainly initiated by the femoral implantation, has been suggested to be the fatal inductor of cardiorespiratory collapse (Modig et al. 1975, Schlag et al. 1976). However, liquid methylmethacrylate monomer, rest monomer from polymerized methylmethacrylate, and even polymethylmethacrylate particles have been shown to cause cytotoxicity in connective tissue, bone and polymorphonuclear cells (Mohr et al. 1958, Sisca et al. 1966, Petty et al. 1978, Pedersen et al. 1983, Horowitz et al. 1991). Exposure of macrophages to particles of polymethylmethacrylate leads to a dose-dependent release of arachidonic acid inflammatory mediators, which is followed by leakage of intracellular LD (Horowitz et al. 1991). These reports are in accordance with our present findings on leukocytes and endothelial cells, where MMA is clearly toxic in concentrations exceeding 5 µg/mL.

The three different cytotoxicity assays used in this study seemed to be equivalent with respect to determination of MMA-induced cytotoxic damage. This observation is in accordance with Wigzell (1965) and Decker et al (1988), who found a close correlation between these cytotoxicity tests.

Doses ≥ 10 µg/mL induced obvious changes in morphology as early as one minute after the addition of MMA to leukocytes and endothelial cells in vitro, which corresponds to the time taken to reach maximal blood MMA concentrations in central venous blood during cemented THR (Svartling et al. 1985, Wenda et al. 1988, Dahl et al. 1992a). Signs of massive cytotox-

icity were evident after 10 min exposure of cells to MMA in vitro. Rotation of the leg following the femoral implantation may cause stagnation of venous blood flow (Stamatakis et al. 1977), which may locally increase the exposure time between the lipophilic MMA and the membranes of blood and endothelial cells in the femoral vein. A half-life of MMA of almost one hour has been demonstrated following the application of a tourniquet during cemented total knee replacement which may thus substantially increase the exposure time to MMA (Svartling et al. 1986) However, even a brief exposure to high concentrations of MMA may within one minute cause toxic damage to the blood cells and endothelium, as shown in this study.

The importance of such local detrimental effects of MMA on endothelial cells is supported by the fact that deep vein thrombosis is found with remarkably higher incidence in patients receiving cemented compared to patients having non-cemented implants and most noticably a higher frequency of proximal thrombi occurs following hip surgery (Stamatakis et al. 1977, Lynch et al. 1988, Francis et al. 1989, Høgevold et al. 1990).

Several patient studies have shown high blood levels of MMA (up to 120 µg/mL) in relation to hip and knee substitute surgery (Svartling 1986, Wenda 1988, Dahl 1992a). It has to be noted that most MMA determinations reported up to now have been performed in the gas phase above plasma/blood (Svartling et al. 1985, Dahl et al. 1992a), which probably underestimates the real liquid phase concentrations due to the highly lipophilic nature of MMA (Mohr 1958, Rijke et al. 1977).

Cytotoxic damage of MMA to leukocytes and endothelial cells occurred in this study at concentrations found in central venous blood during reconstructive joint surgery in 25–100 percent of the patients in different studies (Svartling et al. 1985, Svartling et al. 1986, Wenda et al. 1988, Dahl et al. 1992a). The presumed direct cytotoxic effect of MMA on the endothelium may result in cellular retraction and detachment of endothelial cells which, in turn, may expose subendothelial structures active in promoting coagulation and adherence of blood platelets (Dahl et al. 1992b). Together with impaired fibrinolysis (Dahl et al. 1993) and the possibly disturbed balance between endothelium-derived vasoactive substances (e.g., NO, endothelin), the stage might be prepared for thrombus formation. Sequestration of potentially harmful cells, like monocytes and granulocytes, due to increased adhesiveness, may also compromise the microcirculation, especially in the lungs, and cause damage by releasing proteolytic enzymes and reactive oxygen intermediates. The load of MMA is thus superimposed on existing disturbances of the thrombohemorrhagic balance, and will probably, even in small concentrations, initiate changes contributing to cardiorespiratory and vascular complications.

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