

BACKGROUND: Many papers have focused on the importance of granulocytes in the process of reperfusion and ischemia. Most of the clinical studies measured several parameters of this process during and after coronary angiography, without taking into account the effect of the radiographic contrast media (RCM) used during this procedure.

Materials and methods: We performed a randomized patient study ($n = 37$) to evaluate the effect of ionic and non-ionic RCM on granulocyte adhesion during coronary angiography. We also evaluated the influence of the ionicity and osmolarity of the different substances on granulocyte adhesion molecules in *in vitro* experiments.

Results: The osmolarity of patient serum samples increased from 302 ± 1 to 309 ± 1 mOsm/kg ($p < 0.05$) after infusion of RCM. The CD11b expression in the samples of the non-ionic RCM treated group increased from 221 ± 21 MFI to 377 ± 30 MFI ($p < 0.05$) measured as the absolute mean fluorescence intensity (MFI), yet did not alter significantly in the ionic RCM group. In contrast, the *in vitro* experiments showed a reduction of the CD11b expression from 360 ± 70 MFI to 149 ± 30 MFI ($p < 0.05$) in the ionic RCM group.

Conclusions: The upregulation of adhesion molecules was significantly reduced *in vivo* with ionic RCM, while ionic substances caused opposite effects *in vitro*. This effect should be taken into account when performing leukocyte functional analysis of samples taken during angiography.

Key words: Granulocyte, Monocyte, Adhesion molecules, Radiographic contrast media, Angiography

Influence of ionic and non-ionic radiographic contrast media on leukocyte adhesion molecules

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Introduction

Leukocyte activation has been shown to play an important role in different inflammatory processes. Moreover, activation of leukocytes has been detected in processes not only characterized or accompanied by inflammation; for example, transplantation medicine,¹ shock^{2–4} and ischemia/reperfusion processes.^{5–9}

One of the most studied mechanisms of leukocyte activation is adhesion. Indeed, in order to migrate into tissue, inflammatory cells need to adhere to the vessel wall. This process is linked to an upregulation of adhesion molecules on both leukocytes and the endothelium.^{10–12}

Some studies attempted to analyze whether altered expression of adhesion molecules is detected during ischemia/reperfusion: for instance, in patients submitted to a coronary angiography and percutaneous transluminal coronary angiography.^{13–15} Increased expression of adhesion molecules on leukocytes has been detected in ischemia/reperfusion.

Recently, scientists have become aware of the influence of medication on the activation of leukocytes. Moreover some radiographic contrast media (RCM), which are injected in patients, are known modulators of leukocyte function.¹⁶ However, the influence on the expression of adhesion molecules has not been previously reported. Therefore, we studied the effects of RCM, both assayed *in vitro* and *in vivo*, on the upregulation of granulocyte–adhesion and monocyte–adhesion complexes. We also attempted to discriminate whether these effects were attributable to either RCM ionicity or RCM osmolarity, two characteristics used to classify these agents in four arbitrary groups (Table 1).

Materials and methods

Patient inclusion

The inclusion criteria and study protocol were approved by the ethical committee of the University Hospital of Antwerp. Patients with stable angina,

Table 1. Classification of RCM

	Low osmolarity	High osmolarity
Non-ionic	loversol iopromide lohexol lomeprol	
Ionic	ioxaglate	ioxitalamate Diatrizoate

Overview of the classification of RCM. The classification is based on ionic and osmolar properties.

scheduled for a coronary angiography, were considered eligible for inclusion in the study. Patients were only included after informed consent.

Patients were excluded if (i) any sign of inflammation was present (leukocytosis, recent anamnesis of infection, malignancy, etc.), (ii) anti-inflammatory drugs (steroid or non-steroid) were taken on a regular basis, or (iii) the leukocyte count was above 10^4 cells/mm³ or if a granulocyte percentage above 75% was found upon analysis.

After inclusion, patients were treated at random with ioxaglate (Hexabrix®; Guerbet Codali, Brussels, Belgium) or iopromide (Ultravist®; Schering, Diegem, Belgium). In order to avoid interpretation bias, the used RCM was unknown for the cardiologist.

Healthy volunteers

Several volunteers from the department of biochemistry of the University of Antwerp donated blood for the *in vitro* experiments. The blood samples were checked for leukocyte number and differentiation. Volunteers were excluded according to the same criteria as the patient population.

Catheterization technique

Cardiac catheterization was performed using the Seldinger technique. All patients received a 6 Fr arterial sheath in the left femoral artery. All samples were obtained through the arterial sheath. Heparin (5000 IU) was administered at the beginning of the procedure.

Blood sample collection

Samples were collected using the Vacutainer® technique in 3 ml of ethylenediamine tetraacetic acid (EDTA tubes) (Vacutainer®; Beckton Dickinson, Plymouth, UK) and immediately analyzed. Blood samples were collected before angiography (at baseline) and 5 min after the procedure was completed.

Eleven patients agreed to a third sample collection taken 60 min after completion of the angiography. The latter samples were used to measure the osmolarity and leukocyte activation over a 1-h time course. The samples were analyzed for the mem-

brane density of the leukocyte adhesion complex and for serum osmolarity.

In vitro techniques

Leukocyte adhesion receptor labeling

For a rapid and efficient evaluation of the blood samples, we used the staining procedure described by Macey *et al.*¹⁷ Briefly, whole blood (25 µl) was incubated in the dark room, with 25 µl of 2×10^{-5} M liquid dye solution 751 (LDS 751; Exciton, Dayton, Ohio, USA) at 4°C for 15 min. Subsequently the samples were incubated and labeled with 10 µl of phyco-erythrin-conjugated anti-CD11b antibody (CD11b-PE) (Beckton and Dickinson, Aalst, Belgium) and fluoresceine-isothiocyanate-conjugated anti-CD14 antibody (CD14-FITC) (Beckton and Dickinson). Incubation was performed at room temperature for 10 min. Finally, RPMI-1640 medium (Gibco BRL, Merelbeke, Belgium) buffered with 25 mM of HEPES (Sigma, Bornem, Belgium) was added to a final volume of 500 µl and analyzed on a flow cytometer (FACScan; Beckton Dickinson). CD11b expression, quantified as the mean fluorescence intensity, was measured on granulocytes and monocytes, discriminated by scatter profile and CD14 expression.

Serum osmolarity measurement

Osmolarities were measured using an automated osmometer (Cryomatic®, Model 3C2; Advanced Instruments Inc., Needham Heights, Massachusetts, USA).

In vitro activation assay

Blood samples were collected from healthy volunteers ($n = 5$). The samples were incubated with the two types of RCM used in the catheterization unit of the University Hospital: ioxaglate (HEXABRIX®; Guerbet Codali) and iopromide (ULTRAVIST®; Schering).

After injection in patients, RCM dilutions were estimated during the patient trial, and physiologically acceptable dilutions (290, 310, 330 and 350 mOsm/kg) were tested.

As RCM may have both ionic and osmotic properties, we supplemented phosphate-buffered saline (PBS) solutions (Dulbecco's Phosphate Buffered Saline; Gibco BRL) with either saline or sucrose. Saline solutions have osmotic and highly ionic properties, whereas sucrose is a non-ionic compound. We used both solutions in order to prepare solvents with the same range of osmolarities as RCM. Glucose (G), saline (S), ioxaglate (IX) and iopromide (IP) were tested in the following osmolarities: 290, 310, 330 and 350 mOsm/kg. The Blank PBS was tested in 280 mOsm/kg.

Blood samples of healthy volunteers were collected in 4 ml EDTA tubes (Vacutainer®; Beckton

Dickinson) and were divided in 200 µl aliquots. Each aliquot was incubated with 4 ml of lysis buffer (155 mM NH₄Cl, 10 mM KCO₃, 0.1 mM Na₂EDTA) for 5 min at room temperature.

Subsequently the samples were centrifuged for 15 min at 400 g. The supernatant was discarded and the cell pellet was resuspended in either PBS or one of the osmotic solutions. The incubation with the osmotic solutions was maintained at 37°C during 20 min, an incubation time that induced maximal granulocyte activation as shown in preliminary experiments (data not shown).

The cell suspension was incubated with 10 µl of CD11b-PE and CD14-FITC during 15 min, after which the excess of non-ligated antibody was removed by centrifugation. After resuspension in 500 µl PBS, flow cytometric analysis for adhesion molecules was performed.

Statistical analysis

Statview[®] software (version 5.0; SAS, Cary, North Carolina, USA) was used for all statistical calculations. Corrections for non-parametric analysis were performed (i.e. Mann–Whitney, Kruskal–Wallis and Spearman rank correlation analysis).

Whenever mean values and their range are mentioned, we used the standard error of the mean.

Results

Patient study

A total of 37 patients was included in the study. The population consisted of 19 male subjects (age 61.1 ± 8.5 years) and 18 female subjects (age 66.8 ± 11.5 years). Mean fluorescence signals at baseline showed no difference between the ioxaglate-treated and the iopromide-treated patients (Mann–Whitney $p = 0.72$) (Fig. 1).

Analysis of the samples revealed a significant increase in CD11b intensity as well as osmolarity after 5 min. A subpopulation of 21 patients agreed to donate a third sample collected 60 min after injection of RCM. The results are presented in Fig. 1. Iopromide significantly elevated the CD11b-receptor density in the granulocyte population, 5 min after administration ($p < 0.001$). This effect disappeared after 60 min, and the fluorescence values returned to baseline ($p = 0.81$). The monocyte population showed an analogous trend to elevate the CD11b-receptor density ($p = 0.06$). Monocyte activation also seemed to persist for more than 60 min. Patients treated with ioxaglate showed no significant difference in CD11b-receptor density over time.

The osmolarity of the samples was determined. The measured baseline osmolarity was measured at

302 ± 1 mOsm/kg. Samples taken 5 min after RCM administration showed a significant ($p = 0.003$) elevation of the serum osmolarity (309 ± 1 mOsm/kg). The osmolarity of the samples at baseline and the samples taken 60 min after RCM administration (304 ± 2 mOsm/kg) showed no statistical significant difference. The results are presented in Fig. 2.

In vitro study

To validate the results of the clinical study, we studied the effects of RCM on granulocytes and monocytes *in vitro*. For convenience, the *in vitro* experiments were limited to the two RCMs used in our hospital. Blood samples were collected from healthy volunteers ($n = 5$).

As the maximal osmolarity in patients was estimated to be 309 ± 1 mOsm/kg, we used solutions with an osmolarity varying between 290 and 350 mOsm/kg to study the activation of granulocytes in osmotic media.

To properly evaluate the effect of the solutions we normalized the mean fluorescence values. Granulocytes can exhibit an elevated level of the initial CD11b-receptor density. This form of elevated activity is denoted as 'granulocyte priming'.¹⁸ As the maximal activity (degranulation or upregulation of CD11b) of the granulocyte will not change, a primed granulocyte will have an apparently decreased activity. We normalized the values in such a way that the blank value (PBS without any additives) was correlated with 0% activation and the maximal measured value was expressed as 100% activation.

The results clearly showed a difference between granulocyte and macrophage responses, as observed in the clinical study (Fig. 3). CD11b-receptor density on monocytes is relatively stable, regardless of the degree of ionicity or osmolarity of the RCM. Granulocytes, on the contrary, seem to be more susceptible to these factors.

To evaluate the effect of osmolarity on the activation of granulocytes and monocytes, we prepared a concentration gradient of sucrose-enriched PBS solutions. In order to rule out possible additive effects of ionicity, a comparison was also made with analogous solutions of PBS. Using a saline or sucrose gradient, we could not establish any influence of either the ionicity or osmolarity (Fig. 3), even at 350 mOsm/kg, which is well above the measured osmolarity *in vivo*.

Next to the influence of ionicity and osmolarity we also tested the RCMs. In this way we could evaluate the substance-related effects, regardless of their ionic and osmolar properties. Ioxaglate seems to exert a significant downregulating effect on CD11b-receptors at higher osmolarities; for example, at 330–350 mOsm/kg (hypothesis of no difference).

The effect is most prominent on the activity of granulocytes. Using a Kruskal–Wallis analysis, we

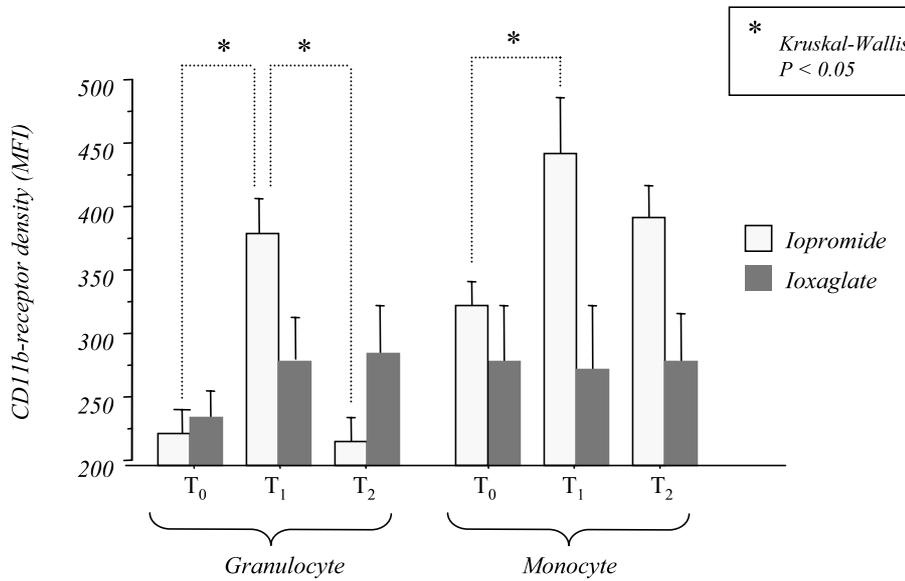


FIG. 1. Changes in CD11b levels on leukocytes after RCM infusion. The mean fluorescence intensity (MFI) of anti-CD11b antibody on granulocytes and monocytes during infusion of iopromide and ioxaglate. T₀, sample taken before the initiation of the procedure; T₁, sample taken 5 min after infusion of RCM; T₂, sample taken 60 min after the infusion of RCM. * Statistically significant differences ($p < 0.05$).

observe a significant difference ($p < 0.05$) between ioxaglate and iopromide (Fig. 3). The effect can be described as an inhibition of the upregulation of CD11b-receptors at the cell membrane. Iopromide does not show such an effect.

To correlate the effect of the ioxaglate concentration (in serum) to its inhibitory potential, a regression analysis with Spearman rank correction was performed (Fig. 4). This analysis produces a spearman rho of 0.7 with an estimated r^2 value of 0.48. No other significant correlations could be found.

Discussion

RCM are frequently used for both diagnostic procedures and clinical research. Altering the X-ray absorption of tissues, vessels and cavities enables the clinician to visualize anatomical structures in the organism, which provides information about organ mass and function, such as revascularization of the coronary arteries.

However, many unwanted pharmacological effects are observed after the use of RCM, ranging from

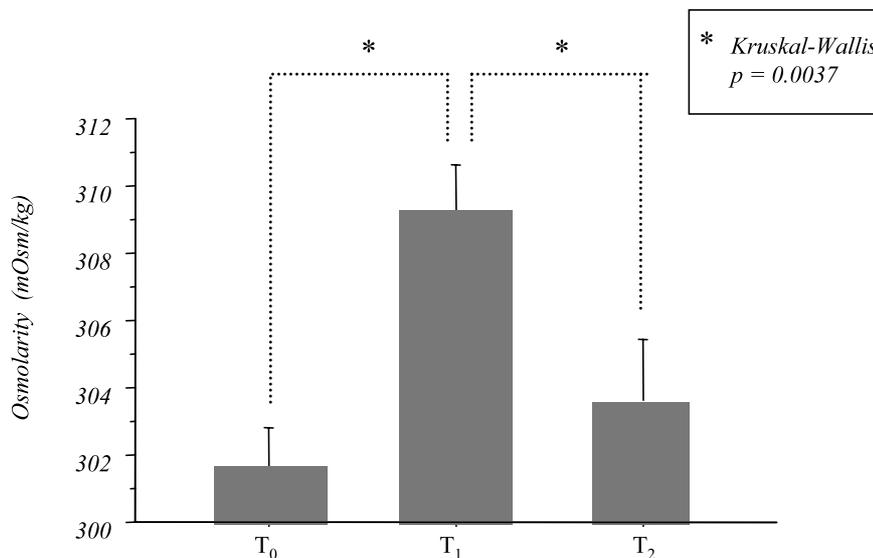


FIG. 2. Transient increase in serum osmolarity after iopromide infusion. T₀, sample taken before the initiation of the procedure; T₁, sample taken 5 min after infusion of RCM; T₂, sample taken 60 min after the infusion of RCM. * Statistically significant differences ($p < 0.05$).

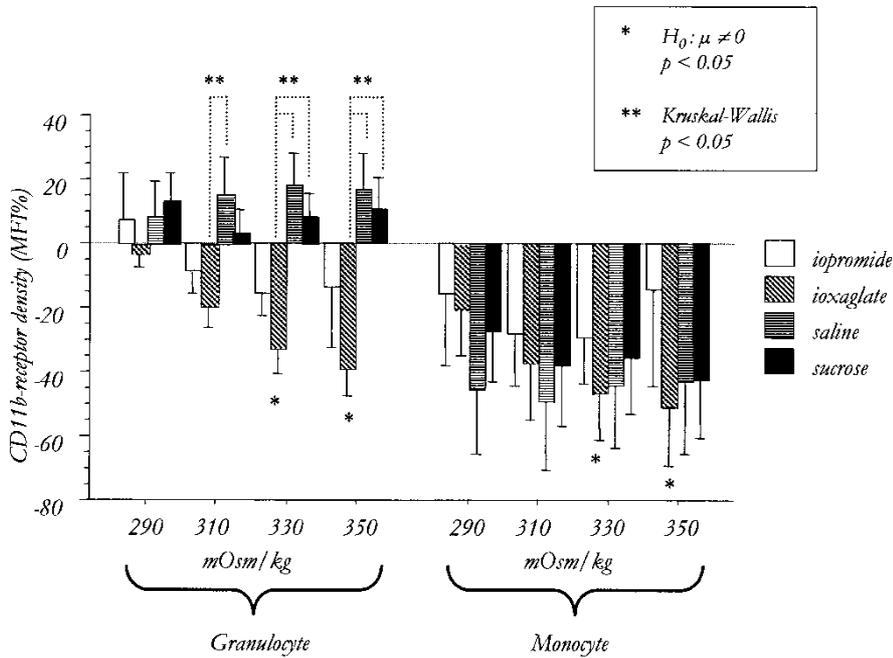


FIG. 3. 'In vitro' effect of osmolarity on CD11b expression. The effect of different solutions of iopromide, ioxaglate, sucrose and saline, with increasing osmolarity, on granulocytes and monocytes. * Statistical significant difference from zero ($p < 0.05$). ** Significant statistical ($p < 0.05$) 'between group' difference. CD11b-receptor density is expressed in% MFI, a value normalized for blank and maximal stimulation.

disruption of the blood-brain barrier¹⁹⁻²¹ to severe anaphylactic reactions.^{22,23} The latter is well studied and many precautions can be taken to prevent²⁴ or treat²⁵ its aftermath. RCMs affect different physiological systems such as the coagulation and arachidonic acid system and may interact with many human cells.

Several publications have covered the problem of an elevated risk for thrombosis with non-ionic substances.²⁶⁻²⁸ *In vitro* experiments have shown that platelets more readily degranulate in the presence of non-ionic substances.²⁹

In our clinical study we observed an elevated CD11b expression after iopromide injection. This 'activating' effect of iopromide, a member of the non-ionic RCM group, has not yet been described in the literature. An increased adhesion to nylon fibers was described by Lang and Lang.³⁰

Yet ioxaglate does not affect the CD11b expression *in vivo*, in contrast to the findings of Feldman *et al.*³¹ The question remains of whether the ionicity or the osmolarity of the substance is actually responsible for this effect.

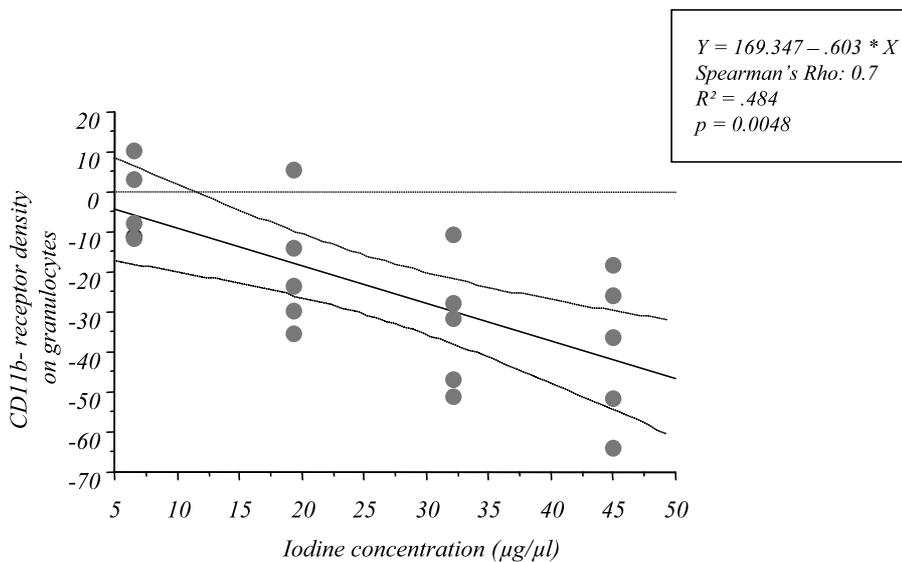


FIG. 4. 'In vitro' effect of ioxaglate concentration on CD11b expression. Linear regression analysis for CD11b expression in% MFI (normalized for blank and maximal value) and iodine concentration of ioxaglate ($\mu\text{g}/\mu\text{l}$). Linear regression was corrected by Spearman rank calculation.

The *in vitro* experiment clearly showed that neither osmolarity nor ionicity affected the activity of the leukocytes in our experiment. Neither could we observe a response to an elevation of the ionicity. Sodium chloride could not evoke any response on the leukocytes. Thus, ionicity alone cannot account for the protective effects as described previously in the literature.³²

From the results of the RCM *in vitro* experiments we may conclude that ioxaglate exerts a down-regulating effect on the adhesion molecules at higher osmolarities. As neither osmolarity, nor ionicity is responsible for this effect, the *in vitro* effect is probably due to the ioxaglate concentration. This *in vitro* effect is more evident on granulocytes than on monocytes and has not yet been previously described. Iopromide showed no significant effect on the leukocytes whatsoever.

At first glance this seems rather contradictory with regard to the *in vivo* experiment. This might well mean that ioxaglate neutralizes a 'catheterization-associated' activation, whereas the protective effect of iopromide is too weak to exert any effect at all.

The results of this study clearly indicate that ionic RCM may well inhibit leukocyte activation. The question remains of whether the choice between ionic and non-ionic RCM is indeed crucial. Several authors implied that the choice between different RCMs is not so important. Regardless of their ionic and/or osmotic properties the overall clinical outcome (in terms of major adverse cardiac events) remains unchanged.^{33,34} Other trials support the use of ionic RCM as being more favorable.³⁵

Several other granulocyte functions are also influenced after contact with RCM, but conflicting data have been published. Following incubation of granulocyte with RCM, both a potentiation and an inhibition of exocytosis (i.e. the release of proteases by degranulation) has been reported.³⁶ Both inhibition³⁷ and increased chemotaxis³⁸ have also been observed. Most papers indicated an inhibition of phagocytosis.³⁹ In general, ionic media are more inhibitory than non-ionic.

Many publications measured the membrane-associated CD11b immediately after angiography or percutaneous transluminal coronary angiography, yet they do not specify the RCM used in their protocol.⁴⁰⁻⁴² The observed *in vivo* effects on granulocyte functions have been described insignificant and transient. However, they should be taken into account when evaluating the early activation *in vivo* during procedures of ischemia/reperfusion. Caution is warranted for the interpretation of experimental results measured within 30 min of RCM administration.

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