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A Comparative *in vitro* Evaluation of Different Therapeutic Protocols for Vascular Graft Infections

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Introduction

Grafting techniques in vascular surgery are frequently complicated by severe infections, associated with mortality and limb-loss rates of 25–40%,[1–4] that occur both early after implantation of grafts and even much later. The severity of these infections is a consequence of the extremely high resistance of bacterial biofilms growing on graft surfaces, due to the production of esopolymeric substances.[5–7] Several approaches have been attempted to overcome this problem, including improvement in aseptic surgical techniques, development of new biomaterials intended to be less suitable for bacterial colonisation, bonding of antibacterial agents to grafts by different means,[8–11] and research for new systemic agents able to disrupt biofilms.[12,13] Each different approach resulted in some improvement in therapeutic outcomes of grafting procedures, but none proved to be a definitive solution.

The most threatening feature of biofilm growth with regard to resistance to antibacterials and host defences is certainly the production of glycocalyx. This was the target of several studies devoted to the development of new therapeutic strategies for prosthetic infections. Recently the results of two studies disclosed two potentially useful strategies to enhance the activity of antibiotics on bacterial biofilms, and namely the use of proteolytic enzymes[13] and the use of macrolides[12] both in association with antibiotics. The present work aimed at evaluating *in vitro* the effect of both proteolytic enzymes and macrolides, in association with different antibiotics and in conjunction, on bacterial biofilms formed on different biomaterials commonly used in vascular surgery.

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Materials and Methods

Throughout the study 15 bacterial strains were used: five were *Staphylococcus aureus* (including reference strain ATCC 6538P), five were *Staphylococcus epidermidis* (including reference strain ATCC 35984), and five were *Pseudomonas aeruginosa*. All strains, except the reference ones, were recently isolated from vascular graft infections, and were identified at the species level using the ATB 32 STAPH and API 20NE systems (Api System, La Balme Les Grottes, Montalieu Vercieu, France).

The following substances were used as antibacterial agents: cefamandole (Sigma Chemical Co., St. Louis, MO, U.S.A.), gentamycin (Sigma), vancomycin (Sigma), ofloxacin (Sigma-Tau, Pomezia, Italy), azithromycin (Pfizer Italia, Rome, Italy), and serratiopeptidase (Takeda Italia, Rome, Italy). All tests in sessile conditions of growth were performed in triplicate in the presence of the following biomaterials: polystyrene beads, 7 mm diameter (The Plastic Ball Co., Chicago, IL, U.S.A.), 7 mm long pieces of both PTFE (W. L. Gore & Associates Inc., Flagstaff, AZ, U.S.A.) and gelseal knitted Dacron grafts (Vascutek Ltd., Inchinnan, Scotland).

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values in three different conditions of growth were determined according to a previously published method[7] as follows: condition A, dilution tubes were inoculated with 10^6 cfu/ml; condition B, dilution tubes containing the biomaterial were inoculated as for condition A; condition C, dilution tubes were inoculated with the biomaterial colonised with 10^6cfu/ml equivalents. All the inocula were prepared from a single overnight broth culture, and were sized by the bioluminescence apparatus Lumac biocounter M2500 (Lumac bv,

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Table 1. MIC and MBC values (μg/ml) of five antibiotics alone and associated with azitromycin at a concentration equal to 25% of the MIC or serratiopeptidase 10 U/ml in three different conditions of growth for *Staphylococcus aureus* SA1511 (comparable results obtained performing tests in conditions B and C with polystyrene, PTFE, or Dacron).

Antibiotic	MIC			MBC		
	A	B	C	A	B	C
Cefamandole	3.12	3.12	3.12	6.25	25	>200
Ofloxacin	0.78	0.78	0.78	1.56	6.25	50
Gentamycin	1.56	1.56	3.12	3.12	6.25	100
Vancomycin	0.78	0.78	3.12	3.12	12.5	50
Azitromycin	0.78	0.78	0.78	3.12	12.5	12.5
Cefamandole*	3.12	3.12	3.12	6.25	25	100
Ofloxacin*	0.78	0.78	0.78	1.56	3.12	25
Gentamycin*	1.56	1.56	3.12	3.12	6.25	50
Vancomycin*	0.78	0.78	3.12	3.12	6.25	12.5
Cefamandole†	3.12	3.12	3.12	6.25	12.5	12.5
Ofloxacin†	0.78	0.78	0.78	1.56	3.12	12.5
Gentamycin†	1.56	1.56	3.12	3.12	3.12	12.5
Vancomycin†	0.78	0.78	1.56	3.12	6.25	6.25
Azitromycin†	0.78	0.78	0.78	3.12	6.25	6.25

* Addition of azitromycin at a concentration equal to 25% MIC.
† Addition of serratiopeptidase 10 U/ml.

Table 2. MIC and MBC values (μg/ml) of five antibiotics alone and associated with azitromycin at a concentration equal to 25% of the MIC or serratiopeptidase 10 U/ml in three different conditions of growth for *Staphylococcus epidermidis* SA1378 (comparable results obtained performing tests in conditions B and C with polystyrene, PTFE, or Dacron).

Antibiotic	MIC			MBC		
	A	B	C	A	B	C
Cefamandole	50	50	50	100	100	>200
Ofloxacin	0.39	0.39	1.56	1.56	3.12	50
Gentamycin	50	50	100	100	100	>200
Vancomycin	6.25	6.25	12.5	25	25	100
Azitromycin	0.19	0.39	0.39	1.56	12.5	50
Cefamandole*	25	25	25	50	50	100
Ofloxacin*	0.39	0.39	0.39	1.56	1.56	6.25
Gentamycin*	50	50	50	50	50	100
Vancomycin*	6.25	6.25	6.25	12.5	12.5	25
Cefamandole†	25	25	25	25	25	50
Ofloxacin†	0.39	0.39	0.39	1.56	1.56	3.12
Gentamycin†	50	50	50	50	50	50
Vancomycin†	6.25	6.25	6.25	12.5	12.5	12.5
Azitromycin†	0.19	0.39	0.39	1.56	3.12	3.12

* Addition of azitromycin at a concentration equal to 25% MIC.
† Addition of serratiopeptidase 10 U/ml.

Landgraaf, The Netherlands) as previously described.[14] Tests were performed using scalar two-fold dilutions of each antibiotic alone and in association with serratiopeptidase 10 U/ml and azitromycin at a concentration equal to 25% of the previously determined MIC value for each test strain, for staphylococci, and at 2 μg/ml for pseudomonas. All tests were performed in Mueller Hinton broth (Difco Lab., MI, U.S.A.).

Results

The MBC values of all antibiotics for all test strains in conditions B and C were much higher than those in condition A (Tables 1–3), and when serratiopeptidase 10 U/ml was added they were significantly reduced. The addition of azitromycin at a concentration equal to 25% of the MIC value (for Gram-positives) and at 2 μg/ml for pseudomonas influenced MBC values in conditions B and C, but less than serratiopeptidase. The most effective association when testing staphylococci was azitromycin and serratiopeptidase, showing only little differences in MBC values in the three experimental conditions (Tables 1 and 2). No differences were observed in MIC and MBC values of all antibiotics, for all test strains in the three experimental conditions with the three different biomaterials tested (Tables 1–3).

Table 3. MIC and MBC values (μg/ml) of for antibiotics alone and associated with azitromycin at a concentration equal to 2 μg/ml, or serratiopeptidase 10 U/ml in three different conditions of growth for *Pseudomonas aeruginosa* PS1347 (comparable results obtained performing tests in conditions B and C with polystyrene, PTFE, or Dacron).

Antibiotic	MIC			MBC		
	A	B	C	A	B	C
Cefamandole	12.5	25	25	25	50	200
Ofloxacin	3.12	3.12	3.12	3.12	12.5	100
Gentamycin	0.78	1.56	1.56	3.12	25	200
Azitromycin	100	200	200	>200	>200	>200
Cefamandole*	12.5	12.5	12.5	25	25	100
Ofloxacin*	3.12	3.12	3.12	3.12	12.5	50
Gentamycin*	0.78	1.56	1.56	3.12	25	100
Cefamandole†	12.5	12.5	12.5	25	25	50
Ofloxacin†	3.12	3.12	3.12	3.12	6.25	12.5
Gentamycin†	0.78	1.56	1.56	3.12	12.5	25
Azitromycin†	100	200	200	200	200	200

* Addition of azitromycin at a concentration equal to 2 μg/ml.
† Addition of serratiopeptidase 10 U/ml.

Discussion

Graft-centred infections are certainly to be considered a serious complication in vascular surgery, both because of mortality and morbidity and for difficulties encountered in their treatment.[1–4] Generally, as a consequence of the bacterial colonisation of grafts, surgery is required to substitute biomaterials in septic condition with a higher risk of failure. Efficient prophylactic and therapeutic protocols are thus needed which overcome the extreme resistance of bacteria

growing in biofilms to antibiotics. Among the strategies potentially useful for this purpose, the search for molecules able to selectively enhance the activity of antibiotics on biofilms seems the most adequate. Recent research showed that both proteolytic enzymes[13] and macrolides[12] can be useful for this purpose. According to the data reported here serratiopeptidase shows an activity higher than azitromycin, and this should be kept in mind when treating infections due to Gram-negative bacteria. Nevertheless, since most vascular graft infections are due to Gram-positive bacteria (staphylococci above all),[15] it would be advisable to associate both serratiopeptidase and azitromycin in the treatment of these infections.

Conclusion

In spite of the fact that specific adhesins for different biomaterials were reported in different bacteria,[16] our data show clearly that both the susceptibility of biofilms to antibiotics and the enhancing activity of serratiopeptidase and macrolides are not significantly influenced by the nature of the colonised biomaterial. These results suggest that these molecules interfere with fundamental metabolic steps involved in the resistance mechanism of biofilms. Clinical studies are in progress to assess the *in vivo* effectiveness of the association of azithromycin and serratiopeptidase in the treatment of vascular graft infections due to Gram-positive bacteria.

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