

Effect of antiadhesive agents on peritoneal carcinomatosis in an experimental model

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Background: Auto-crosslinked polysaccharide hyaluronan-based solution (Hyalobarrier[®]-gel) prevents postoperative adhesions. However, its effect on tumour growth is still unknown. The aim of the present study was therefore to investigate the impact on survival of intra-abdominally administered Hyalobarrier[®]-gel, native hyaluronan (HA) and hyaluronan/carboxymethylcellulose (HA/CMC), after intraperitoneal tumour implantation.

Methods: After receiving an intraperitoneal inoculum of the human HT29 colorectal cell line, 615 athymic nude mice were assigned randomly to five groups: groups 1 and 2 received Hyalobarrier[®]-gel 20 mg/ml ($n = 124$) and 40 mg/ml ($n = 126$) respectively; groups 3 and 4 received HA ($n = 120$) and HA/CMC film (SeptrafilmTM) ($n = 123$) respectively. The survival of each treated group was compared with that of group 5, the control, which had no treatment ($n = 122$).

Results: As 34 of the 615 mice were not eligible, 581 animals were considered for the analysis. At 120 days, 136 animals (23.4 per cent) were still alive. At autopsy there was macroscopic absence of tumour in 75 cases (12.9 per cent). No statistically significant differences were found between the treatment and the control groups with respect to postoperative death and absence of tumour implantation. There was no difference in survival rate between the control group and groups treated with Hyalobarrier[®]-gel, HA or HA/CMC.

Conclusion: Hyalobarrier[®]-gel, HA and HA/CMC had no negative impact on the survival rate in mice that received an intraperitoneal implantation of HT29 colorectal human tumour cells.

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Introduction

Postoperative intraperitoneal surgical adhesions, common after abdominal surgical procedures, consist of fibrous connective tissue bridges between tissues or internal organs that are normally separate. The pathophysiology of adhesion formation is unclear. It is probable that serosal injury, bleeding and fibrin deposition in the abdominal cavity, together with reduced fibrinolytic activity, are the main causative factors. Postoperative adhesions may have severe clinical consequences, including intestinal obstruction, chronic pelvic pain and infertility¹, the treatment of which significantly increases healthcare costs².

A wide variety of approaches, including use of steroids, non-steroidal anti-inflammatory drugs and minimally invasive surgical techniques, has been used in an attempt to prevent adhesions. However, biodegradable barriers

appear to be the most promising tools available for keeping adjacent organs separate following surgery³. The efficacy of these barriers has been demonstrated in clinical trials^{4,5}. Recently, a new hyaluronan (HA) derivative, auto-crosslinked polysaccharide (ACP), was developed for the prevention of postoperative adhesions. ACP, in the form of a gel (Hyalobarrier[®]-gel; Fidia Advanced Biopolymers, Abano Terme, Italy), displays physicochemical properties different from those of native HA, for example, higher viscosity⁶. Its beneficial effects – reduction in the incidence of adhesions and their severity – have been demonstrated in different animal models of abdominopelvic and gynaecological surgery^{7–9}.

However, as the relationship between intraperitoneal HA and HA-based devices and the development of primary tumours and metastasis is unclear, and because there

is a lack of experimental and clinical evidence, use in patients with abdominal cancer may be questionable. The main endpoint of this *in vivo* experimental study was to evaluate the effect of two different preparations of Hyalobarrier[®]-gel, used intraperitoneally to prevent adhesions, on the survival rate of mice given a tumour inoculum intraperitoneally. As a secondary endpoint, the same hypothesis was tested for native hyaluronan (HA) and hyaluronan/carboxymethylcellulose (HA/CMC) film.

Material and methods

Preparations of materials

Hyalobarrier[®]-gel was prepared by hydrating an ACP powder in 5-ml glass syringes, and sterilizing the preparation with steam. Gel concentrations of 2 and 4 per cent were obtained. HA/CMC film (Septrafilm[™]; Genzyme Corp., Cambridge, MA, USA) was sterilized by γ -radiation. All treatment materials were handled in aseptic conditions before placement at surgery.

Human colorectal tumour cell lines

The human colorectal cancer cell line HT29 was cultured in RPMI (Gibco BRL, Paisley, UK) and supplemented with L-glutamine 2 mmol/l, 4-(2-hydroxy-ethyl)-1-piperazine ethanesulfonic acid 10 mmol/l, streptomycin 150 units/ml, penicillin 200 units/ml and 10 per cent heat-inactivated fetal calf serum (PAA Laboratories, Linz, Austria).

Animals

The animal model consisted of 615 athymic nude (*nu/nu*) male mice (weight 20–25 g; aged 5–6 weeks) supplied by Charles River Laboratories (Calco, Como, Italy), and housed under controlled environmental conditions. This model is widely used for experimental oncology studies. Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies.

Statistical design and sample size

According to the null hypothesis for the primary endpoint, the postoperative administration of Hyalobarrier[®]-gel in two concentrations (20 and 40 mg/ml) should negatively affect the survival of athymic mice that have received a HT29 human tumour cell inoculum. The suggested negative effect of Hyalobarrier[®]-gel 20 mg/ml and Hyalobarrier[®]-gel 40 mg/ml was considered equivalent to that of no treatment (control) if the treated group's

probability of surviving at 90 days was no less than 15 per cent of that observed in the untreated mice (one-tail test). Since two comparisons were planned (control *versus* Hyalobarrier[®]-gel 20 mg/ml and control *versus* Hyalobarrier[®]-gel 40 mg/ml), the sample size was corrected with the Bonferroni method in order to ensure a level of total significance of 90 per cent ($\alpha = 0.1$). It was estimated that a sample size of at least 116 mice per group would achieve a power of 80 per cent ($\beta = 0.2$).

Surgical procedures and treatment

The surgical procedures described in this study were in accordance with Standard Operating Procedures and the procedure described in the Laboratory Notebook. Animal care and surgery were performed under the Italian Ministry of Health regulation no. 116/92. The animals were anaesthetized with an intraperitoneal injection (0.2 ml per 10 g) of a solution containing ketamine hydrochloride (Ketavet; Gellini Pharmaceuticals, Aprilia, Italy) 10 per cent and xylazine hydrochloride (Rompun[®]; Bayer, Leverkusen, Germany) 2 per cent. The abdomen was then prepared with iodine solution and a vertical 1.5-cm midline laparotomy performed. After injecting a single dose of HT29 tumour cells (0.3 ml of suspension containing 166×10^5 cells/ml) into the abdomen (approximately 5.0×10^4 cells/mouse), the animals were assigned randomly to five groups. Groups 1 and 2 received Hyalobarrier[®]-gel 20 mg/ml (a single dose of 0.5 ml ACP gel; $n = 124$) and 40 mg/ml (a single dose of 0.2 ml ACP gel; $n = 126$) respectively; groups 3 and 4 received HA (a single dose of 0.2 ml; $n = 120$) and HA/CMC film (1.5×1.5 cm Septrafilm[™]; $n = 123$) respectively; and the control group (group 5) had no treatment ($n = 122$). The amount of biomaterial administered was tenfold the dosage suggested for humans.

At the end of the surgical procedure, the midline wound was closed using 5/0 Dexon continuous suture (Davis-Geck, Catania, Italy). The mice were given 0.1 ml nefopam analgesic (Nefam[®]; Farma-Biagini, Naples, Italy), and allowed to feed and drink freely immediately after surgery.

Monitoring

Death and the presence of clinical signs of disease were monitored daily by the animal housing operators and weekly by the veterinary surgeon. Any cases of anomalous behaviour or disease were recorded in the Veterinary Examination Request sheets provided by the Animal Care Service secretariat. The United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR)¹⁰ guidelines

Table 1 Characteristics of the treatment groups and the control group

Treatment	<i>n</i>	Postoperative deaths	Not eligible	Eligible	No tumour at necroscopy
Hyalobarrier [®] -gel 20 mg/ml	124	10		114	18
Hyalobarrier [®] -gel 40 mg/ml	126	13		113	22
HA 40 mg/ml	120	3		117	9
HA/CMC film	123	3		120	16
Control	122	3	2	117	10
Total	615	32 (5.2)	2 (0.3)	581 (94.5)	75 (12.2)

Values in parentheses are percentages. HA, native hyaluronan; CMC, carboxymethylcellulose.

for the welfare of animals in experimental tumours were followed strictly.

Assessment of body-weight and tumour weight

Each animal was weighed twice weekly from the day of surgery. If the loss of body-weight was more than 20 per cent with respect to the initial weight, the animal was killed following the UKCCCR guidelines. After each animal's death, the macroscopic abdominal tumour was removed and weighed. The median and interquartile range (i.q.r.) for body-weight and tumour weight in each treatment group were calculated.

Assessment of survival rate

Animals were observed for 4 months after operation. Survival was considered as the interval between the date of operation and the date of the event (animal's death or suppression). The percentage of survivors for each group was recorded daily and at the end of the study.

Statistical analysis

Survival curves for the study groups and control group were constructed using the Kaplan–Meier method and the null hypothesis of non-equivalence was tested using a modified log rank test¹¹. The magnitude of the difference between 90-day survival rates in treated and untreated groups was given as a 90 per cent confidence interval. The same method was applied for the secondary endpoint of the study. Medians were compared using Wilcoxon's rank sum test, and percentages were compared using the χ^2 test. Statistical significance was set at $P < 0.05$. Analysis of data was performed using the SAS statistical package (release 8.00; SAS Institute, Cary, North Carolina, USA).

Results

Thirty-two (5.2 per cent) of the 615 mice died within 5 days of operation, and two other mice were not eligible (females); the remaining 581 animals were considered for analysis. At 4 months, 136 animals (23.4 per cent) were still alive, and were killed according to the protocol design. Autopsy analysis showed a macroscopic absence of tumour implant in 75 cases (12.9 per cent). No statistically significant differences were found between the treatment groups and the control group with respect to postoperative deaths and absence of tumour implantation (*Table 1*).

Survival rate

The difference between the 90-day survival rate of the Hyalobarrier[®]-gel 20 mg/ml and the control group, and between the Hyalobarrier[®]-gel 40 mg/ml and the control group was close to zero. Comparing the curves (modified log rank test), neither treatment was found to be inferior to the other (*Fig. 1*). The upper limit of the 90 per cent confidence interval, which identifies the maximum acceptable difference to allow the conclusion that the treatments are equivalent, was always lower than 15 per cent (*Table 2*). Comparable findings were also made for the native HA and HA/CMC groups (*Fig. 1* and *Table 2*).

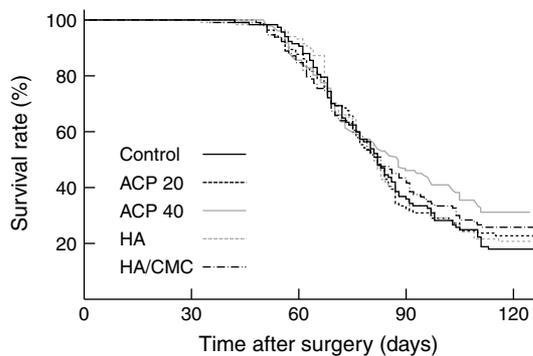
Tumour weight

The median (i.q.r.) tumour weights after animal suppression or death were 2.9 (1.9–4.1), 3.1 (2.5–3.9), 3.5 (2.7–4.4), 2.8 (2.0–4.0) and 3.4 (2.4–4.4) g for Hyalobarrier[®]-gel 20 mg/ml, Hyalobarrier[®]-gel 40 mg/ml, native HA, HA/CMC and control groups respectively. No statistically significant differences were found between the treatment groups and controls in weight of tumour at death.

Table 2 Comparison between the survival rates of the treatment groups and that of the control group

Treatment	n	Survival rate at 90 days	Modified log rank test	P*	90% c.i. for difference at 90 days†
Control	117	0.36			
Hyalobarrier®-gel 20 mg/ml	114	0.33	-3.378	< 0.001	-0.068, 0.137
Hyalobarrier®-gel 40 mg/ml	113	0.46	-4.822	< 0.001	-0.208, 0.005
HA	117	0.35	-3.393	< 0.001	-0.095, 0.112
HA/CMC	120	0.42	-3.698	< 0.001	-0.162, 0.047

HA, native hyaluronan; HA/CMC, hyaluronan/carboxymethylcellulose; c.i., confidence interval. *Probability that treatments have a negative impact on survival. †The survival rate at 90 days of treated groups was never less than 15 per cent (one-tail test).



No. at risk	Control	ACP 20	ACP 40	HA	HA/CMC
Control	117	117	107	43	21
ACP 20	114	114	100	38	25
ACP 40	113	113	97	53	35
HA	117	117	109	43	24
HA/CMC	120	120	102	52	31

Fig. 1 Kaplan–Meier survival curves comparing groups treated with a Hyalobarrier®-gel (auto-crosslinked polysaccharide (ACP) gel) at concentrations of 20 mg/ml (ACP 20; n = 114) and 40 mg/ml (ACP 40; n = 113), native hyaluronan (HA, n = 117) or hyaluronan/carboxymethylcellulose film (HA/CMC; n = 120) and the control group (n = 117)

Discussion

When used after operation in a laparoscopic rabbit model, Hyalobarrier®-gel significantly reduced adhesion formation with respect to the untreated group⁷. However, the effects of these bioresorbable barrier devices on cancer cell implantation and metastasis are still unknown, and the surgical community questions their use in patients with colorectal cancer. No clinical study has been conducted to address these issues, and studies on the efficacy of bioresorbable barrier devices have included only patients with benign diseases.

To investigate whether Hyalobarrier®-gel, native HA and HA/CMC film might interfere with tumour implantation and development, a study was conducted

in nude athymic mice that first received an inoculum of HT29 human tumour cells and then underwent treatment with four bioresorbable barrier devices (Hyalobarrier®-gel 20 mg/ml, Hyalobarrier®-gel 40 mg/ml, native HA, and HA/CMC film). The survival rates of the treated groups were compared with that of a control group. The survival curves of the treated mice were equivalent to those of the untreated mice. No statistically significant differences were found between groups with respect to the number of mice that developed cancer or median tumour weight.

Results from other experimental studies that have addressed this issue are contradictory. HA may interfere with tumour growth and proliferation in many ways. It may provide a stable matrix for cancer cell proliferation. Colorectal tumour cells that express HA appear to have an increased invasive and metastatic potential¹². Colorectal cancer cells may produce hyaluronidase that, by breaking down HA, produces oligosaccharides, thus stimulating angiogenesis, an essential step in the metastatic process¹³. Another possible interference between HA-based devices and tumour growth stems from the fact that HA is a major ligand of CD44. This is a family of type I transmembrane glycoproteins that are widely expressed on a variety of cells of epithelial, mesenchymal and haematopoietic origin. CD44 and its variants are present in a wide range of human tumours. Grossly enhanced and deregulated expression of CD44 is evident during colorectal cancer development^{14,15}. Intraperitoneal HA administration may improve tumour cell adhesion by providing numerous sites for HA–CD44 interaction.

Using colorectal tumour cell lines with different CD44 expression, Tan *et al.*¹⁶ found that HA enhanced the metastatic potential *in vivo* and *in vitro*. *In vivo*, there was a significantly higher total tumour nodule count in the peritoneal cavity in HA-treated rats than in the control group. Moreover, in their *in vitro* studies, HA significantly increased tumour cell proliferation and

motility in a dose-dependent fashion, whereas CMC had no significant effect. However, while HA-induced tumour cell motility appeared to be CD44 receptor dependent, HA-induced tumour cell proliferation was CD44 receptor independent. The authors therefore concluded that HA might enhance intraperitoneal tumour growth.

Other *in vivo* and *in vitro* studies reach opposite conclusions. Haverlag *et al.*¹⁷ found that (Sepracoat™; Genzyme Corp., Cambridge, MA, USA) a solution of sodium hyaluronate in phosphate-buffered saline, inhibited adhesion of CC-531 colonic carcinoma cells to the mesothelium *in vitro*, but had no appreciable effect on intra-abdominal tumour growth in an *in vivo* rat model. Using a nude mouse model, Burgess *et al.*¹⁸ found that neither Seprafilm™ bioresorbable membrane nor Sepracoat™ coating solution increased the incidence of tumour growth following intraperitoneal KM12-L4 human colon cancer cell implantation with respect to an untreated control group. Underwood *et al.*¹⁹ showed that Seprafilm™ had no influence on colon cancer cell implantation at abdominal laparoscopic wound sites in a hamster model.

Importantly, the HT29 colon cancer cells used in the present study had a very high level of CD44 receptor expression, as revealed by cytofluorimetric analysis (data not shown), suggesting that this glycoprotein did not play a relevant role in tumour growth, under the present experimental conditions. Several possible factors may explain the discrepancies: the animal models and the cell lines used; the methods employed to assess tumour growth and metastatic development; and sample sizes and experimental designs. Both Tan *et al.*¹⁶ and Haverlag *et al.*¹⁷ reached their conclusions using very small groups of animals, and tumours were assessed only by visual inspection. Underwood *et al.*¹⁹ had an excessive rate of implantation of tumour cells across all groups, thus diminishing the sensitivity for detection of any subtle effects of the bioresorbable membrane. In the current study the authors tried to obviate these potential weaknesses by using an adequate sample size, and survival as a more objective endpoint. These experimental conditions allow more reliable conclusions to be drawn.

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