Does the concomitant intra-arterial injection of asialoerythropoietin and edaravone mitigate ischaemic mucosal damage after acute superior mesenteric artery thromboembolism in a rabbit autologous fibrin clot model?

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ABSTRACT. To increase the survival rate of patients with acute superior mesenteric artery thromboembolism (ASMAT) treated by catheter thrombolysis, we examined the effects of delivering edaravone and asialoerythropoietin, agents with tissue-protective activities, using a rabbit autologous fibrin clot ASMAT model. Japanese white rabbits ($n=32$) were randomly separated into four equal groups. 45 min after introducing autologous fibrin clot, Group U received urokinase and heparin; Group E received urokinase and heparin plus edaravone; Group A received urokinase and heparin plus asialoerythropoietin; and Group EA received urokinase, heparin and edaravone plus asialoerythropoietin via a catheter. The intestines were removed 6 h later and intestinal mucosal damage was scored using the Park's injury score. Survival time was assessed. Average mucosal injury was 5.78 ± 1.52 (Group U), 2.88 ± 0.72 (Group E), 1.90 ± 1.23 (Group A) and 1.18 ± 1.25 (Group EA). The degree of mucosal injury was significantly lower in Group EA than in Groups U and E ($p<0.05$). Conversely, there was no significant difference between Group A and Group EA, or between Group A and Group E. The survival times were 31.50 ± 13.30 h (Group U), 51.00 ± 24.74 h (Group E), 48.00 ± 16.97 h (Group A) and 82 ± 51.07 h (Group EA); the difference among the four groups was not significant. In conclusion, the concomitant administration of asialoerythropoietin and edaravone reduced mucosal membrane injury significantly compared with edaravone alone. However, to improve the survival of ASMAT rabbit models, the delivery of an appropriate dose of asialoerythropoietin is required, together with the development of methods to assess peripheral recanalisation.

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The mortality rate associated with acute superior mesenteric artery thromboembolism (ASMAT) remains high, despite improved diagnostic and therapeutic techniques. Approximately 50% of patients with ASMAT die after thrombolysis or suction or surgical thrombectomy [1]. An important factor in the high mortality of ASMAT patients is the generation of active oxygen by ischaemia-reperfusion (I-R) injury. We and others [2–4] have reported that the administration of oxygen free radical scavengers, such as superoxide dismutase or dimethylthiourea, reduces the degree of I-R injury in animal models. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-1; norphenazone; MCI-186), a potent scavenger of hydroxyl and peroxy radicals, inhibits lipid peroxidation via its antioxidant activity. Animal studies on the mitigation of small intestine damage found that edaravone decreases the degree of I-R-mediated mucosal tissue injury [5–7]. Elsewhere, we showed that, in a rabbit ASMAT model that introduced

autologous fibrin clots, thrombolysis via the intra-arterial administration of edaravone and urokinase contributed to a reduction in the degree of I-R injury to the small intestinal mucosa [4]. We attributed the failure of edaravone to improve the survival time to inadequate thrombolysis in some areas of the intestinal mucosa and postulated that damage caused by ischaemia rather than reperfusion resulted in critical intestinal injury (e.g. infarction) [4]. Asialoerythropoietin, a desialated form of erythropoietin that is non-hematopoietic but appears to retain extra-hematopoietic effects, is an analogous substance to erythropoietin [8, 9] and exerts tissue-protective activities [9–13]. Mori et al [11] recently reported the protective effect of asialoerythropoietin against I-R injury and apoptosis in the small intestine of rats. We hypothesised that concomitant intra-arterial administration of asialoerythropoietin and edaravone may improve survival time because asialoerythropoietin protects inadequately reperfused areas and edaravone prevents I-R injury.

In the current study, we used the catheter technique applied in the clinical setting and a rabbit autologous fibrin clot ASMAT model to examine the effects of edaravone and asialoerythropoietin delivered intra-arterially via the superior mesenteric artery (SMA).

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Methods and materials

Evaluation of mucosal injury

The study protocol was approved by our Animal Experimentation Committee; all experiments were performed according to the Animal Care Guidelines of our institution. The rabbit SMA thrombosis model was established as reported elsewhere [4]. We randomly divided 32 adult Japanese white rabbits into four equal groups: Group U, the reperfusion group, received urokinase only; Group E received urokinase plus edaravone; Group A received urokinase plus asialoerythropoietin; and Group EA received urokinase plus edaravone and asialoerythropoietin. All rabbits were placed under general anaesthesia with medetomidine hydrochloride (0.1 mg kg^{-1} intramuscularly) (DomitorTM; Meiji Seika Co. Ltd, Tokyo, Japan) and ketamine hydrochloride (25 mg kg^{-1}) (Ketalar 50TM; Sankyo Yell Yakuhin Co. Ltd, Tokyo, Japan). The right femoral artery was surgically exposed and a 4 Fr sheath (Clinical Supply Co. Ltd, Gifu, Japan) was inserted using a cut-down method. A 4 Fr cobra-type catheter (Clinical Supply Co. Ltd.) was introduced into the trunk of the SMA and arteriography was performed by manually injecting 2 ml of iopamidol (Bayer HealthCare, Osaka, Japan). Contrast medium (iopamidol) was added to 10 ml of blood extracted via the catheter, and autologous blood clots (6 ml), produced by a 45 min incubation at 36°C , were delivered via a 4-Fr cobra-type catheter to produce a rabbit model of ASMAT. Arteriography was performed 5 min and 10 min later by manually injecting 2 ml of iopamidol to assess recanalisation. In animals exhibiting recanalisation, we repeated the clot delivery procedure.

To ascertain the absence of recanalisation, repeat arteriography was performed after 45 min (2 ml of iopamidol delivered manually). Group U received both 6000 IU of urokinase (Mitsubishi Tanabe Pharma Corp., Osaka, Japan) and 250 IU of heparin (Mitsubishi Tanabe Pharma Corp.) in saline (Otsuka Pharmaceutical Co. Ltd, Tokyo, Japan) via the catheter. Group E was treated with urokinase (6000 IU), heparin (250 IU) and edaravone (0.5 mg kg^{-1}), whereas Group A was treated with the same amounts of urokinase and heparin plus a single dose (1000 IU kg^{-1}) of asialoerythropoietin. Lastly, Group EA was injected with the same amounts of urokinase and heparin plus edaravone (0.5 mg kg^{-1}) and a single dose of asialoerythropoietin (1000 IU kg^{-1}). After 10 min, the blood flow recovery was confirmed arteriographically by manually injecting 2 ml of iopamidol.

Histopathological evaluation

6 h after reperfusion, all rabbits were sacrificed by injecting 5 ml of pentobarbital (Dainippon Sumitomo Pharma Co. Ltd, Tokyo, Japan) into the heart; the small intestine located 20 cm from the pylorus to the terminal ileum was harvested. For random specimen extraction, each intestine was divided into five equal parts and a 2 cm segment was excised from the proximal side. Each segment was immersed for 24 h in 10% buffered formalin and then embedded in paraffin. A $4\text{-}\mu\text{m}$ -thick slice of the sagittal section was deparaffinised and

stained with haematoxylin and eosin (H&E). Using the system of Park et al [14], the degree of injury in the intestinal tissue, where damage is greatest, was scored on a scale from 0 to 8: where 0 = normal mucosa, 1 = subepithelial space at villus tip, 2 = greater extension in the subepithelial space, 3 = epithelial lifting along the villus sides, 4 = denuded villi, 5 = loss of villus tissue, 6 = crypt layer infarction, 7 = transmucosal infarction and 8 = transmural infarction. The degree of mucosal injury was recorded as average, maximal or minimal.

Evaluation of the survival time

Four rabbits from each group were selected randomly and observed for one week to assess their survival time. These animals received saline and enrofloxacin (2.5%) every 24 h by injection.

Statistical analysis

We used SPSS II for Windows (SPSS Japan Inc, Tokyo, Japan) for analysis. Significant differences in the average, maximal and minimal mucosal injury scores and in the survival times were evaluated with the Tukey honestly significant difference (HSD) test. A p -value <0.05 was considered significant.

Result

Microscopic mucosal evaluation

In Group U, we observed denuded villi, loss of villus tissue and crypt layer, and transmucosal and transmural infarction. Conversely, Groups E, A and EA manifested villus defluxion and flattening of the lamina propria mucosae in some tissue sections. Most specimens appeared to be only slightly damaged; the mucosa, subepithelial space at the villus tip and the more extended subepithelial space were normal (Figure 1).

Average mucosal injury score

The Park injury score was 5.78 ± 1.52 in Group U, 2.88 ± 0.72 in Group E, 1.90 ± 1.23 in Group A and 1.18 ± 1.25 in Group EA. Mucosal damage was significantly lower in Group EA than in Groups U and E.

Maximal mucosal injury score

The Park injury score was 6.63 ± 1.30 in Group U, 6.00 ± 1.41 in Group E, 4.88 ± 2.23 in Group A and 3.25 ± 3.28 in Group EA. A significant difference was observed only between Group U and Group EA.

Minimal mucosal injury score

The Park injury score was 4.38 ± 2.88 in Group U, 0.25 ± 0.46 in Group E and 0.00 ± 0.00 in Groups A and EA. Groups E, A and EA were significantly different from Group U. There was no significant difference among Groups E, A and EA.

Figure 1. (a) Group U: denuded villi, loss of villus tissue and crypt layer, transmucosal and transmural infarction are all observed. (b–d) Groups E, A and EA: villus defluxion and flattening of the lamina propria mucosae area are noted in some sections ($\times 100$).

Survival times

The survival time was 31.50 ± 13.30 h in Group U, 51 ± 24.74 h in Group E, 48.00 ± 16.97 h in Group A and 82.00 ± 51.06 h in Group EA. Although the survival time was prolonged in some Group EA rabbits, there was no overall significant difference among the four groups.

Discussion

The present study showed that, in rabbits, thrombolysis through the concomitant administration of asialoerythropoietin and edaravone prevents mucosal injury by ASMAT more effectively than thrombolysis with edaravone alone. However, it failed to increase the survival time.

With respect to the average mucosal injury score, the concomitant intra-arterial administration of asialoerythropoietin (Group AE) significantly reduced the degree of mucosal damage compared with Groups U and E. Conversely, the maximal mucosal injury score was not significantly different among Groups E, A and EA. These observations indicate that, although the concomitant

delivery of edaravone and asialoerythropoietin lessens the degree of overall damage to the intestinal mucosa, it does not protect against critical mucosal injury. We obtained similar results for the survival time. Although one rabbit in Group EA survived for one week, most of the remaining rabbits in all four groups died fewer than four days after administration. Asialoerythropoietin did not contribute markedly to long-term survival.

We explain our findings as follows. At catheter thrombolysis, tiny peripheral vessels that cannot be checked by fluoroscopy failed to recanalise or recanalised inadequately. In other words, asialoerythropoietin did not reach some areas of the mucosa. Alternatively, the asialoerythropoietin dose was too low to be effective throughout the entire intestine. Consequently, the damage to mucosal tissue exposed to inadequate amounts of asialoerythropoietin progressed to necrosis, resulting in the death of the animals.

Either way, our findings indicate that thrombolysis through the concomitant administration of asialoerythropoietin and edaravone was more effective in the prevention of mucosal injury than the delivery of edaravone alone.

To our knowledge, this is the first study in a rabbit autologous fibrin clot ASMAT model that investigated the

effectiveness of the intra-arterial administration of both edaravone and asialoerythropoietin. However, there are some limitations to our study. First, we introduced autologous fibrin clots that were produced *in vitro*, which may not be identical to thrombi developed under pathophysiological conditions. Second, our study was based on a small number of rabbits because our experiments exposed the animal to significant distress. Third, we assessed the treatment effects by digital subtraction angiography to imitate the clinical setting, although CT studies are required to evaluate emboli on the mucosal peripheral level. Fourth, we did not prepare a control group. As our primary purpose was to assess the additive effect of asialoerythropoietin, we kept the number of rabbits exposed to the procedures as low as possible, and, therefore, we did not include a non-treated group.

In conclusion, our experiments showed that the concomitant intra-arterial administration of asialoerythropoietin and edaravone lessened the degree of mucosal injury in our rabbit ASMAT model. In efforts to prolong the survival time of patients ASMAT, studies are under way in our laboratory to determine the required dose of asialoerythropoietin and to develop methods to demonstrate inadequate recanalisation of tiny peripheral vessels.

Practical applications

The mortality rate is especially high in elderly patients with SMA thrombosis because many of these individuals have concurrent cardiac disease. Our catheter-based treatment lessens the burden on the elderly subpopulation and can be performed easily. Therefore, it may help to improve their survival rate. Furthermore, our catheter-based method slows the progress of existing intestinal tract necrosis and shortens both the operation time and the time required for post-operative recovery.

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