Concise report

Effects of an anti-platelet drug on the prevention of steroid-induced osteonecrosis in rabbits

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Abstract

Objective. To investigate the effects of an anti-platelet drug (clopidogrel) on the prevention of steroid-induced osteonecrosis (ON) in rabbits.

Methods. Adult male Japanese white rabbits were divided into two groups and treated as follows: one group received daily clopidogrel mixed in normal saline (AP; n = 35), the other received only normal saline (NS; n = 30). One week after the administration, all rabbits were injected once intramuscularly with 20 mg/kg of methylprednisolone acetate into the right gluteus medius muscle. Three weeks after, both the femora and humeri were examined histopathologically for the presence of ON. The platelet aggregation assay and hematological examinations were performed before and after the steroid injection.

Results. The incidence of ON in the AP group (48.5%) was significantly lower than that observed in the NS group (73.3%). The platelet aggregations in the AP group were significantly inhibited by the administration of clopidogrel. The levels of total cholesterol and triglycerides showed no significant differences between the AP and NS group.

Conclusion. The present experimental study demonstrated that the administration of an anti-platelet drug prevented steroid-induced ON in rabbits and that platelet aggregation seems to be one of the possible factors involved in the pathogenesis of steroid-induced ON.

Key words: anti-platelet drug, osteonecrosis, corticosteroid, animal model.

Introduction

Osteonecrosis (ON) has been reported to occur in patients who have received CS treatment for underlying diseases such as SLE and renal transplantation [1]. The natural history of untreated ON of the femoral head generally involves a progressive collapse that often requires surgical treatments. Therefore, the prevention of ON is an optional strategy for such patients.

The precise pathogenesis of CS-induced ON remains unclear. Several possible factors involved in the pathogenesis of ON have been suggested based on human and animal studies, including coagulation abnormalities, hyperlipidaemia, oxidative stress and endothelial dysfunction [2–6]. These factors were thought to be related to an interruption of the bone vascular supply and result in bone ischaemia. In some arterial ischaemic events, including cardiovascular diseases and brain strokes, many reports have demonstrated the crucial role of platelet aggregation resulting from dysfunction of the endothelium [7]. Anti-platelet drugs have therefore been widely used to prevent these ischaemic events. However, to our knowledge, there have been no studies assessing the effects of an anti-platelet drug for the prevention of steroid-induced ON.

Adenosine diphosphate (ADP) released from damaged vessels and red blood cells induces platelet aggregation through activation of glycoprotein Iib and Iila and adhesion using von Willebrand factor. Clopidogrel is a novel anti-platelet drug that selectively and irreversibly inhibits the binding of ADP to its receptor on platelets [8]. Several studies have reported the efficacy and safety of clopidogrel compared with aspirin or ticlopidine in reducing the risk of ischaemic events [9, 10]. We therefore examined whether clopidogrel could reduce the risk...
of steroid-induced ON in an experimental rabbit model and evaluated how CSs and clopidogrel influence haematological parameters.

**Methods**

We utilized a rabbit model of steroid-induced ON [2]. All experiments were conducted in accordance with the Guidelines for Animal Experiments Committee of our institution, Japanese law (no. 105), and notification (no. 6) of the Japanese government.

**Animals**

We studied 65 adults (defined as animals with closed growth plates) male Japanese white rabbits (Kyudo, Saga, Japan) ranging in age from 28 to 32 weeks. The mean (s.d.) body weight of rabbits was 3490 (180) g. Animals were housed at the Animal Center of Kyushu University and maintained on a standard laboratory diet and water.

**Treatment**

The rabbits were randomly divided into two groups as follows: one group received 5 mg/kg of body weight of clopidogrel (Sanofi-Aventis, Paris, France) mixed with normal saline (5 ml/kg/day), which was administered intra-gastrically through a rubber gastric tube into the stomach (AP group; n = 35). The other group received normal saline alone (5 ml/kg/day) intra-gastrically (NS group; n = 30). All rabbits were given the drug or normal saline once daily for 3 weeks. One week after the initiation of the study, all rabbits were injected intramuscularly into the right gluteus medius muscle with 20 mg/kg of body weight of methylprednisolone acetate (MPSL; Pfizer, New York, NY, USA). Three weeks after the administration of drugs, all rabbits were killed and tissue specimens were prepared as previously described [2]. The steroid-induced ON rabbit model has been reported to be reproduced precisely [2–5, 11, 12].

**Evaluation of ON**

The diagnosis of ON was determined at 3 weeks after the administration of drugs (2 weeks after the steroid injection) as previously reported [3, 5, 11, 12]. The specimens stained with haematoxylin and eosin, which were obtained from the proximal one-third and distal condyle of both the femora and humeri (eight regions), were examined histologically for the presence of ON. The presence of ON was assessed blindly by four authors (R.Y., T.Y., G.M. and S.I.) [2, 3, 12] on the basis of the presence of diffuse empty lacunae or pyknotic nuclei of osteocytes within the bone trabeculae, accompanied by surrounding bone marrow cell necrosis. If the diagnoses differed between the four investigators, a consensus was reached by discussion of the histological findings without knowledge of the group from which the specimen was obtained. Rabbits with at least one ON lesion among the eight areas were therefore considered to have ON.

**Platelet aggregation assay**

To examine the anti-platelet effect of clopidogrel, heparinized rabbit whole-blood samples at 0–3 weeks after the initiation of treatment were measured according to the screen filtration pressure method using WBA Carma (IMI, Saitama, Japan) [13]. The samples (200 μl) in reaction tubes were stirred at 5 g and incubated for 1 min at 40°C, then with 22.2 μl of ADP (LMS, Tokyo, Japan) solution for 2 min at 40°C. Using a 3.7-mm-diameter syringe containing screen microsieves made of nickel, with 300 openings of 20 × 20 μm² in a 0.8-mm diameter area, the samples were placed under a vacuum to detect aggregation pressure at a rate of 200 μl/6.4 s. The pressure rates (%) were measured in four concentrations of ADP (final concentration = 0, 1.0, 4.0 and 16.0 μM). The concentration of ADP causing a 50% increase in the pressure rate was calculated and applied as the platelet aggregation threshold index (PATI), which indicates the anti-platelet effect of drugs.

**Examination of laboratory data**

We collected 5 ml of blood samples from the auricular arteries while the animals were in a fasting state in the early morning. Samples were obtained just before the initiation of anti-platelet drug or normal saline treatment (Week 0) and at Weeks 1, 2 and 3 after the beginning of treatment. Biochemical and haematological evaluations were made for plasma levels of triglycerides and total cholesterol, as well as number of platelets.

**Statistical analysis**

Data were expressed as the means (s.d.). The numbers of ON-positive rabbits and rates of ON-positive regions were compared using the chi-square test. Differences in laboratory data between the AP and NS groups were analysed by repeated-measures analysis of variance (MANOVA). If an interaction between two factors of a group and time point was significant in repeated MANOVA, a simple main effect test on the group difference at the same time point was applied. Temporal changes in laboratory data were analysed in each group by the Wilcoxon signed-rank test. Statistical analyses were performed using the JMP 8.0 software package (SAS Institute, Cary, NC, USA). P < 0.05 was considered to be statistically significant.

**Results**

**Incidence of ON**

Two rabbits in the AP group died. One of the rabbits died for an unknown reason at 1 day after the MPSL injection, and the other died due to bleeding from the injured site caused by the insertion of a rubber gastric tube 3 days after the injection. No rabbits died in the NS group.

The incidence of ON in the AP group was 48.5% (16 out of 33 rabbits), whereas that in the NS group was 73.3% (22 out of 30 rabbits). There was a significant difference in the incidence of ON between the AP and NS
groups ($P = 0.042$). The rate of proximal femur involved in ON in the AP group (42.4%) was significantly lower than that in the NS group (70.0%) ($P = 0.027$). In other regions, there was no significant difference of the rate of ON between the AP and NS groups.

The histological appearances and severities of ON were similar in the AP and NS groups (Fig. 1A and B). In the metaphysis and diaphysis of both groups, yellowish ON areas were observed, in which an accumulation of bone marrow cell debris was seen and the bone trabeculae showed empty lacunae.

**Platelet aggregation assay**

The PATI in the AP group showed a significant increasing tendency after the administration of clopidogrel (Weeks 0–1, $P = 0.001$; Weeks 1–2, $P < 0.0001$; Weeks 2–3, $P < 0.0001$), whereas that in the NS group showed no significant change (Fig. 2A). The PATI in the AP group was significantly higher than that in the NS group by repeated MANOVA ($P < 0.0001$) with a significant interaction between the group and time point ($P < 0.0001$). Based on the simple main effect test, the PATI between two groups were found to be significantly different at Weeks 1, 2 and 3 after the drug administration (Week 1, $P = 0.043$; Week 2, $P < 0.0001$; Week 3, $P < 0.0001$).

**Examination of the laboratory data**

No significant differences were seen in the levels of triglycerides and total cholesterol between the AP and NS groups ($P = 0.44$ and 0.47) (Fig. 2B and C). After MPSL injection, these levels showed similar patterns in both groups. After MPSL injection, the numbers of platelets were significantly decreased in two groups ($P = 0.0006$ and 0.020) (Fig. 2D). However, the number of platelets in the AP group remained significantly higher than in the NS group ($P = 0.027$), especially at Weeks 1 and 2 ($P = 0.015$ and 0.0046).

**Discussion**

Anti-thrombotic drugs are generally classified into two types, anti-platelet drugs and anti-coagulant drugs. Anti-platelet drugs are mainly used for the prevention of arterial ischaemic diseases, such as cardiovascular diseases or atherothrombotic stroke through inhibition of platelet aggregation [7]. On the other hand, anti-coagulant drugs are mainly used for prevention of thrombotic diseases, such as coagulation due to atrial fibrillation or deep venous thrombosis by inhibition of clot formation [14]. Anti-coagulant drugs, such as warfarin, have been reported to have preventive effects against steroid-induced ON [3]; however, there are no reports regarding the effects of anti-platelet drugs on the prevention of steroid-induced ON.

In arterial ischaemic diseases, many reports have demonstrated the crucial role of platelet aggregation resulting from the dysfunction of the endothelium [7]. Kang et al. [15] reported an epidemiological study in which the incidence of coronary heart disease was significantly higher in patients who had avascular necrosis. They suggested that common risk factors between avascular necrosis and coronary heart disease, such as smoking, obesity and hyperlipidaemia, may have been responsible. Kerachian et al. [6] reported that CSs led to the dysfunction of the endothelial cell as well as the regional endothelial bed in the bone vascular system, causing steroid-induced ON. In the present study, we demonstrated the possibility that clopidogrel may be effective for the prevention of steroid-induced ON resulting from the inhibition of platelet aggregation.

Several mechanisms have been implicated in the pathogenesis of steroid-induced ON. Hyperlipidaemia has been identified as an important possible contributor to ON. Jaffe et al. [16] suggested that steroid-induced hyperlipidaemia could increase the amount of fat within the femoral head, elevate intra-cortical pressure and lead to sinusoidal collapse. Wang et al. [17] have undertaken studies to show how altered lipid metabolism might lead to...
However, in the present study, the levels of triglycerides and total cholesterol showed no differences between the two groups, which at least indicated that the reduction of the incidence of steroid-induced ON was partly achieved by the inhibition of platelet aggregation without any significant effect on the lipid metabolism.

The major limitation of the present study is that there are differences in drug effects and bioavailability between humans and animals. We showed a sufficient effect on the inhibition of platelet aggregation in the animal study based on the previous reports [13]. Secondly, anti-platelet drugs have several side effects, such as excessive bleeding, thrombotic thrombocytopenic purpura and agranulocytosis. In the present study, none of the animals showed such side effects. Clopidogrel was recommended as one of the first choice of drug for management of cardiovascular diseases in the American Heart Association guideline in 2002 because of the efficacy and safety [10]. In addition, recent study reported that concomitant usage of proton-pump inhibitors with clopidogrel reduced the rate of gastrointestinal bleeding without blunting the efficacy [18]. Thirdly, in the present study, the anti-platelet drug alone could not achieve the complete prevention of steroid-induced ON. Some experimental studies have suggested other effective drugs for the prevention of steroid-induced ON, including warfarin, statin and vitamin E [3, 4, 11]. These results may indicate that steroid-induced ON has a multi-factorial pathogenesis including contributions of hyperlipidaemia, coagulation abnormalities, oxidative stress and platelet aggregation. In conclusion, clopidogrel may be useful as one of the possible candidates for the prevention of steroid-induced ON, and further animal studies and eventual human clinical trials are warranted.
Rheumatology key messages

- The administration of an anti-platelet drug prevented steroid-induced ON in experimental rabbit model.
- Platelet aggregation may be one of the possible factors involved in the pathogenesis of steroid-induced ON.

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