Effects of Ipriflavone on Mammary Carcinogenesis, Uterine Adenomyosis and Bone Mineral Density of Tibia in Mice

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Abstract

Ipriflavone (7-isopropoxy-isoflavone), a derivative of a natural isoflavone isolated from alfalfa (*Medicago setiva* L.), seems to enhance the formation of bone-like nodules in rat bone marrow cell cultures by stimulating osteoblast differentiation. A derivative of ipriflavone enhanced bone formation via an estrogen receptor-mediated pathway in ovariectomized rats. Virgin mice of the SHN strain have a high incidence of mammary tumors and uterine adenomyosis, the genesis of which needs a hormonal environment associated with prolactin and ovarian sex steroids. We investigated the effects of ipriflavone on mammary carcinogenesis, uterine adenomyosis, and bone in virgin SHN mice. Treatment with high-dose ipriflavone slightly lowered the incidence and number of mammary tumors though not significantly, and significantly decreased tibial bone mineral density with an elevation in plasma alkaline phosphatase activity in the intact mice. It was suggested that ipriflavone plays an anti-estrogenic role via the estrogen receptors in animals with an estrous cycle.

Key words —— ipriflavone, phytoestrogen, breast cancer, uterine adenomyosis, bone

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Introduction

Ipriflavone (IF: 7-isopropoxy-isoflavone), a derivative of a natural isoflavone isolated from alfalfa (Medicago setiva L.), has been used to inhibit bone resorption in Japan since 1988[1-2]. IF seems to act not only as an inhibitor of osteoclast activity[3] but also as an enhancer of osteoblast activity[2]. IF appeared to enhance the formation of bone-like nodules in rat bone marrow cell cultures by stimulating osteoblast differentiation[4]. A dietary supplement of IF enhanced the bone mineral density (BMD) of mandibular trabecular and cortical bone in growing rats[5]. TAK-778, a derivative of IF, was reported to enhance bone formation via an estrogen receptor-mediated pathway in ovariectomized rats[6]. Taken together, IF seems to act as a phytoestrogen.

Virgin mice of the SHN strain have a high incidence of mammary tumors and uterine adenomyosis, the genesis of which requires a hormonal environment associated with prolactin and ovarian sex steroids[7-8]. In the present study, we investigated the effects of IF on mammary carcinogenesis, uterine adenomyosis, and the bone mineral density of tibia in virgin SHN mice.

Materials and Methods

Virgin mice of the SHN strain maintained in the animal research center of Tokyo Medical and Dental University (Tokyo, Japan) were used. This strain, a gift from Professor Dr. Takao Mori, Department of Biological

Sciences, Graduate School of Science, University of Tokyo, has a high incidence of mammary tumors and uterine adenomyosis.

The mice were housed in plastic cages with wood shavings under controlled temperature (24 ± 0.5℃) and lighting (12 h of light from 0600 to 1800 h) conditions, and given free access to a commercial diet (CE-2, CLEA Japan, Tokyo, Japan) and tap water. All procedures used on the mice were described in detail in a protocol that was approved by the Animal Care and Use Committee of the Graduate School of Medicine, Tokyo Medical and Dental University, and all experiments conformed to the regulations described in the U.S. National Institutes of Health (NIH) Guide to the Care and Use of Laboratory Animals.

At 71 days of age, the animals were divided into two experimental groups of 10 mice each. For the control group, 24 mice were prepared. In the experimen-tal groups, CE-2 containing ipriflavone (IF: 7-isopropoxy-isoflavone, $C_{18}H_{16}O_3$, MW 280.32, Osten®, Takeda Pharmaceutical Co., Ltd., Osaka, Japan: two dosages of 100 mg and 1 g in 1 kg of diet) was provided for 210 days. In the control group, CE-2 alone was given for 210 days. Assuming the daily intake of diet to be 3 g in a 30 g mouse, the daily intake of IF was calcu-lated to be 0.3 mg and 3.0 mg at low and high doses, respectively.

When a palpable mammary tumor appeared, it was re-moved under anesthesia and fixed in a 10 % formaldehyde buffer solution (pH 7.2). Mice were anesthesized with ether, bled by cardiac punc-ture, and sacrificed by cervical dislocation, and their organs were removed and weighed just before death by diseases or at 280 days of age. The plasma samples obtained were stored at -80℃ for the later determination of plasma levels of biochemical markers. Removed uteri and mammary tumors were fixed in a 10 % formaldehyde buffer solution (pH 7.2), embedded in paraffin, and prepared as 5-μm serial sections, then stained with Mayer's hematoxylin and eosin for histological examination. Each tibia was fixed in 99.5 % ethanol and stored for the later determination of bone mineral density (BMD).

BMD was determined by dual energy absorptiometry (DXA). Total BMD (Ca mg/cm^2) of the entire tibia was measured by DXA (Aloka, DCS-600, Tokyo, Japan) as bone mineral content (BMC) /bone area. The BMD of the proximal metaphysis of the tibia was also measured by DXA as BMC/bone width (Ca mg/cm^2), i.e. part of the trabecular bone.

Data were expressed as the mean ± S.E.M. (standard error of the mean). Statistical analyses were carried out using a one-way analysis of variance (ANOVA) and the unpaired *t*-test. The p values less than 0.05 were considered to be statistically significant.

Results

There were no differences in body growth among the groups (**Table 1**). There were few differences in the wet weights of the liver, spleen, kidneys, and adrenals. The wet weight of the ovaries tended to decrease with the dose of IF though not significantly.

There were few differences in the plasma levels of albumin, glucose, creatinine, total cholesterol (TCh), triglyceride (TG), and free fatty acid (FFA), and the activities of aspartate aminotransferase (AST), among the groups (**Table 2**). On the other hand, the activities of lactate dehydrogenase (LD) and alkaline phosphatase (ALP), and the plasma levels of blood urea nitrogen (BUN), were increased by high-dose IF ($p < 0.05$).

Tumor incidence and the number of mammary tumors in the high-dose IF group tended to be lowered to two-third and a half of those of the control, respectively, though not significantly (**Table 3**). There were no differences in the latent periods of the mammary tumors between the groups. There were few differences in the numbers of survivors and the incidence of uterine adenomyosis at the age of 40 weeks.

Bone mineral density (BMD) was significantly reduced to 94.6% and 92.3% of levels in the control by the intake of high-dose IF in the whole tibia ($p < 0.01$) and the proximal metaphysis of the tibia ($p < 0.05$), respectively (**Figure 1**).

Discussion

Virgin mice of the SHN strain have a high incidence of mammary cancer and uterine adenomyosis[9-10], the genesis of which requires a hormonal environment

Table 1 Body growth and organ weights

Groups	Control	Ipriflavone 0.1 g/kg diet	Ipriflavone 1 g/kg diet
(n)	(24)	(10)	(10)
Body weight			
Final BW (g)	30.9 ± 0.4	29.5 ± 0.5	31.4 ± 0.4
Growth (%)	110.5 ± 5.6	103.4 ± 1.0	102.8 ± 1.4
Organ weight (mg)			
Liver/g BW	44.0 ± 0.8	46.1 ± 1.1	46.0 ± 1.0
Spleen/g BW	75.1 ± 3.7	66.7 ± 1.5	76.1 ± 4.6
Kidneys/g BW	10.8 ± 0.2	9.9 ± 0.2*	10.3 ± 0.3
Adrenals/100g BW	39.7 ± 1.4	45.0 ± 2.4	40.7 ± 2.2
Ovaries/100g BW	77.7 ± 5.7	71.0 ± 4.0	63.9 ± 4.5

Data are means ± SEM.
*Significantly different from that of the control: $p < 0.05$

Table 2 Plasma biochemical markers

Groups	Control	Ipriflavone 0.1 g/kg diet	Ipriflavone 1 g/kg diet
(n)	(24)	(10)	(10)
Albumin (g/dl)	2.09 ± 0.11	1.85 ± 0.06	1.89 ± 0.07
Glucose (mg/dl)	105.4 ± 8.6	122.0 ± 3.9	100.1 ± 6.3
AST (IU/l)	71.8 ± 14.1	71.8 ± 14.7	37.6 ± 4.7
LD (IU/l)	10.3 ± 2.2	12.3 ± 4.0	27.1 ± 9.9*
ALP (IU/l)	61.1 ± 4.2	66.3 ± 5.7	79.3 ± 5.6*
BUN (mg/dl)	24.5 ± 1.3	28.3 ± 1.7	30.3 ± 1.4*
Creatinine (mg/dl)	0.16 ± 0.01	0.16 ± 0.02	0.16 ± 0.01
TCh (mg/dl)	44.3 ± 4.3	42.3 ± 4.4	44.6 ± 3.1
TG (mg/dl)	20.3 ± 3.1	20.1 ± 4.2	17.9 ± 3.3
FFA (μEQ/l)	1027 ± 64	1063 ± 81	952 ± 73

Data are means ± SEM.
*Significantly different from that of the control: $p < 0.05$

Table 3 Mammary tumors and uterine adenomyosis

Groups	Control	Ipriflavone 0.1 g/kg diet	Ipriflavone 1 g/kg diet
(n)	(24)	(10)	(10)
Mammary tumors			
Incidence	11/24	5/10	3/10
Number	14/24	6/10	3/10
Latent periods (w)	33.4 ± 1.1	34.2 ± 3.0	33.0 ± 3.5
Survival (40W of age)	20/24	8/10	8/10
Uterine adenomyosis			
Incidence	22/24	9/10	10/10

Data are means ± SEM.

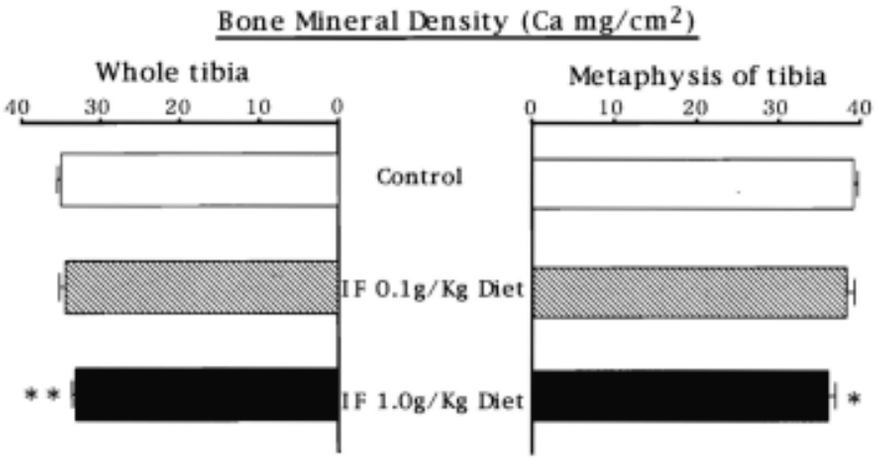

Fig.1 Bone mineral density (BMD: Ca mg/cm^2) of the tibia.
Data are the mean ± SEM in whole tibia (left-sided) and proximal metaphysis of the tibia (right-sided). Right and left upper white bars for the control, a couple of middle bars with gray-stripe pattern for mice supplemented with low-dose of ipriflavone and bilateral lower black bars for mice supplemented with high-dose of ipriflavone.
Asterisks, ** and *, represents significant difference from each corresponding control at $p < 0.01$ and 0.05, respectively.

associated with prolactin and ovarian sex steroids[7-8]. A derivative of IF, TAK-778, induced bone regeneration and stimulated fracture healing in an animal model[11], increased bone formation via an estrogen receptor-mediated pathway in ovariec- tomized rats[6], and enhanced ALP activity in rat bone marrow cell cultures[12]. In the present study, treatment with high-dose IF slightly lowered the incidence and number of mammary tumors though not significantly, and significantly decreased tibial BMD values with an elevation in plasma ALP activity in "intact" mice, but not ovariectomized mice. It is suggested that IF acts via the estrogen receptors in animals with an estrous cycle, i.e. its action lowered the gonadotropin secretion from anterior pituitary gland, followed by the circulating levels of endogenous estrogens, thus leading to the reduction in the incidence of mammary tumors and BMD values of tibia. From the present results, it may be recommended that IF should be used in the treatment of osteoporotic bone disorders in only postmenopausal women, with monitoring of the incidence of breast cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SAS made substantial contributions to the study's conception and the analysis of the data, carried out the animal experiments, and was involved in drafting the manuscript. KIK performed the statistical analysis. OKA, SUZ, and KUD participated in drafting the manuscript. SAK conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All the authors read and approved the final draft.

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マウス乳癌，子宮腺筋症および脛骨骨密度に与えるイプリフラボンの影響

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要旨

マメ科ウマゴヤシ属の多年草アルファルファ（和名：紫馬肥やし）は，牛などに牧草として与えられる．これより分離抽出されたイソフラボン誘導体イプリフラボンは，骨芽細胞の分化誘導を刺激して，ラット培養骨髄細胞で，類骨結節形成を促すとされ，また，あるイプリフラボン誘導体は，卵巣摘出ラットで，エストロゲン受容体を介して骨吸収を抑制する事が報告されている．臨床でも骨粗鬆症治療薬として認められている．今回，乳腺腫瘍・子宮腺筋症自然発生系マウスであるSHN系雌マウスを用いて，乳腺癌化，子宮腺筋症および脛骨骨密度に与えるイプリフラボンの影響について検討した．イプリフラボン高濃度投与群では，乳腺腫瘍の発生と数量を有意ではないものの低める傾向を示し，血中アルカリフォスファターゼ活性の上昇を伴いながら，脛骨骨密度を有意に減少させた．このことは，去勢動物ではなく，性周期を有する動物においては，イプリフラボンがエストロゲン受容体を介して抗エストロゲン作用を示すことが示唆された．以上の結果，イプリフラボンは乳癌検診を前提に，閉経後婦人の骨粗鬆症治療にのみ使用されるべきかもしれない．

キーワード

イプリフラボン，植物エストロゲン，乳癌，子宮腺筋症，骨密度