Title: Exercise effects on methylation of *ASC* gene

Abstract

Chronic moderate exercise has been reported to reduce pro-inflammatory cytokines. To analyze the molecular mechanisms by which training exerts these effects, the epigenetic influences of age and exercise on the *ASC* gene, which is responsible for IL-1β and IL-18 secretion, were investigated by *ASC* gene methylation. Further, the relationship between carcinogenesis and exercise, methylation of the *p15* tumor suppressive gene was analyzed as well. High-intensity interval walking exercise, consisting of 3-minute low-intensity walking at 40% of peak aerobic capacity followed by a 3-minute high-intensity walking period above 70% of peak aerobic capacity, was continued for 6 months. Peripheral blood DNA extracts from young control (n=34), older control (n=153), and older exercise (n=230) groups were then analyzed by pyrosequencing for DNA methylation. Methylation of *ASC* decreased significantly with age (young control vs. older control, $p<.01$), which is indicative of an age-dependent increase in ASC expression. Compared to the older control group, the degree of *ASC* methylation was higher in the older exercise group (older control vs. older exercise:, $p<.01$) and presumably lower ASC expression. Neither exercise nor age affected the methylation of the *p15*. In summary, chronic moderate exercise appears to attenuate the age-dependent decrease in ASC methylation, implying suppression of excess pro-inflammatory cytokines through reduction of ASC expression.

Introduction

The relationship between exercise and susceptibility to infection has been modeled in the form of a “J”-shaped [18] or “S”-shaped curve [13]. These models imply that although moderate activity may enhance immune function, excessive amounts of prolonged and highly intensive exercise may actually impair function. Other anti-inflammatory effects mediated through the suppression of pro-inflammatory cytokines were also reported to be possible benefits of moderate exercise.

Chronic inflammation caused by excess pro-inflammatory cytokines, such as TNF-α, IL-β, and IL-6, plays a prominent role in the development and progression of several age- and inactivity-related diseases. The relationship between chronic moderate exercise and reduced inflammation has been proven in several reports, which also identified some of the molecular mechanisms involved.

Cytosolic pathogen receptors have also garnered attention in the field of inflammation and innate immunity. One such molecule, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), is an adopter molecule consisting of a caspase recruit domain (CARD) and a pyrin domain (PYD) that was first revealed in our laboratory as a proapoptotic protein [15, 24]. ASC functions as a mediator in the cytosol-type inflammatory signaling pathway, as well as a downstream molecule in toll-like receptors (TLRs) signaling. It has been established to activate procaspase-1 [22] and process proIL-1β and proIL-18 to IL-1β and IL-18, respectively [14, 28] and ultimately lead to the initiation of innate immunity. *ASC* gene expression is recognized to be modified epigenetically, i.e. is down regulated by the methylation of its CpG island surrounding exon 1, and the methylation was inversely correlated with ASC protein expression [11, 29]. Here, we investigated how ASC activity is epigenetically influenced by age or chronic moderate exercise by assessing the methylation of the CpG islands in the *ASC* gene using bisulfate genomic sequencing methods.

Another representative gene whose expression is epigenetically changed by methylation is the tumor suppressor gene *p15* [1]. Since the relationship between exercise and cancer has also been reported [27], *P15* was added to the present analysis.

Taken together, the main purpose of this study is to investigate whether such epigenetic changes as methylation of the *ASC* and *p15* genes are influenced by age or chronic moderate exercise, namely a 6-month high-intensity interval walking regimen [17], in older subjects.

Materials and Methods

Subjects and Exercise application

A total of 436 (142 male and 294 female) healthy subjects in two groups who participated in The Health-promotion Program for Elderly People in Matsumoto (Jukunen Taiiku Daigaku) were object of our present research. One was the older control group (n=162, age 40-87, 64.9 ± 7.4 years) and the other was the older exercise group (n=274, age 41-86, 65.7 ± 7.4 years). For the young control group, 37 university students were recruited from Matsumoto University (age 18-22, 19.4 ± 0.9 years).

Subjects following the high-intensity interval walking regimens were instructed to repeat several sets of 3-minute low-intensity walking at 40% of peak aerobic capacity followed by a 3-minute high-intensity walking period above 70% of peak aerobic capacity for 6 months. Exercise intensity and time were supervised by accelrometry (Jukudai mate, Kissei Comtec Co., Ltd, Nagano). Mean walking time was 3.9 ± 1.2 days per week, 52.2 ± 18.5 minutes per day. The lowest acceptable training frequency was 2days/week and training time was 26min/day. Close monitoring was maintained for the first month of the regimen until subjects could learn high-intensity interval walking. Every two weeks, the subjects visited a local office to transfer the data from their tracking devices to a central server at the administrative center for automatic analysis and reporting. Trainers used these reports to track daily walking intensity and other parameters and to instruct subjects on how to best achieve exercise target levels. Dropouts were excluded from the beginning. Clinical data of the subjects are shown in Table 1.

This study has been approved by the Committee on the Use of Human Research Subjects of Shinshu University School of Medicine and Matsumoto University, and also performed in accordance with the ethical standards of the IJSM [12]. Participant's consent was received.

Bisulfite Treatment

Genomic DNA was extracted from the peripheral blood of control groups and subjects after a 6-month exercise period using a QIAamp DNA Midi Kit (QIAGEN, MD, USA. Blood sampling was performed preprandially at approximately 8:30 in the morning around one week after the last session of interval walking. From that, 1 μg of DNA was treated with sodium bisulfite using a CpGenome DNA Modification Kit CpGemone (Chemicon, Temecula, CA, USA) according to manufacturer's instructions.

PCR and Pyrosequencing

Bisulfite-modified DNA was resuspended in a total volume of 50 μl elution buffer and 0.3 μl, for *ASC,* amplified by a 1st-round of PCR (Go Taq DNA polymerase, Promega, Madison, WI, USA) and then adjusted to a final volume of 20 μl with TE buffer. The 2nd-round (nested) amplification was performed using 1 μl of the first-round PCR product, and then the 2nd-round PCR product was adjusted to a final volume of 50 μl with TE buffer. For *p15*, 5 μl bisulfite modified DNA was amplified by PCR and then adjusted to a final volume of 70 μl with TE buffer. Seven sites in the CpG island of *ASC* designated by Conway *et al.* [5] (fig 1) and 6 sites in *p15* cited by Bollati et al. [1] were selected for pyrosequencing analysis. The primers and PRC conditions used are shown in Table 2.

DNA methylation was quantified using bisulfite-PCR and pyrosequencing of 50 μl or 70 μl of PCR products [7] (Biotage, Summit Pharmaceuticals Intl. Corp., Tokyo, Japan). Individual *ASC* gene pyrosequencing data, whose all 7 sites were judged as successful, were subjected to statistical analysis. After adjustments for technical error, the size of the older control group, older exercise group, and young control group used for statistic analysis was 153, 230 and 34, respectively.

Statistical Analysis

The Student's t-test and ANOVA followed by the post-hoc test were used to test for differences in methylation levels among categorical variables. An F-test for ANOVA was performed to evaluate for normal distribution of test data. Correlation of clinical data with

ASC methylation was estimated using Pearson's correlation coefficient, and association of data with *ASC* methylation was evaluated by both stepwise (backward elimination) and forced entry multivariable linear regression analysis with SPSS version 11.0J software (SPSS Japan Inc., Japan). A *P* value of less than 0.05 was considered statistically significant. Values were expressed as mean ± standard deviation (SD) of the mean for characteristic data and mean ± standard error (SE) of the mean for methylation data.

Results

Clinical data of subjects

The values of body mass index (BMI) and body fat were significantly lower ($p<.05$), and VO_2max was higher ($p<.05$) in young control group than in older control group. As far as within older groups, there was a significant difference in glucose value ($p<.05$) between the older control group and older exercise group (Table 1). When pre- (no methylation data) and post-exercise clinical data in the older exercise group were compared, only VO_2max was found to be significantly increased by exercise (21.9 ± 4.3 to 23.8 ± 5.1 ml/kg/min, $p<.01$).

ASC Findings

A schematic view of the *ASC g*ene region analyzed in this study is indicated in fig 1. *ASC* methylation in the older control group (5.33 ± 0.14 %) was significantly lower than that in the young control group (6.73 ± 0.38 %, $p<.01$, fig 2), indicating that expression of ASC increased age-dependently. Analysis using Pearson's correlation coefficient showed that age ($p<.001$), BMI ($p<.005$), body fat ($p<.001$), and VO_2max ($p<.001$) were correlated with *ASC* methylation among clinical data in Table 1 between young control and older control groups. Since only age (standardized multivariate regression coefficient beta -0.422, $p<.001$; $R^2=0.178$, $p<.001$) remained by stepwise with backward elimination multivariable linear regression analysis for the association with ASC methylation, forced entry multivariable linear regression analysis was then applied. The analysis also revealed that *ASC* methylation was associated with age (beta -0.396, $p<.001$), but not BMI (beta 0.009, $p<.920$), body fat (beta -0.175, $p<.059$), or VO_2max (beta -0.045, $p<.696$, Table 3), indicating that age was the strongest contributor to *ASC* methylation.

Next, the influence of chronic moderate exercise on *ASC* methylation was investigated in older subjects. As shown in fig 3 Left, methylation was higher in the exercise group than in the control group at all 7 sites in the CpG island. At 6 of these sites, the difference was significant ($p<.05$ or $p<.01$). The mean methylation value of the 7 sites together was

significantly higher in the older exercise group (6.29 ± 0.26 %) than in the older control group (5.33 ± 0.14 %, $p<.01$, fig 3 Right), clearly indicative of a decrease in ASC expression by chronic moderate exercise. Examination of data using Pearson's correlation coefficient showed that exercise days ($p<.030$) was correlated with *ASC* methylation between older control and older exercise groups, whereas other clinical data in Table 1, including glucose ($p<.083$), were not.

The relationship between *ASC* methylation, age, and exercise is summarized in fig 4. The mean methylation value was significantly higher in the older exercise group than in the older control group and approximated a value closer to that of the young control group, indicating that the methylation state of *ASC* was markedly improved by the exercise.

p15 Findings

The methylation rate did not change age-dependently in the case of *p15*, nor was it affected by the 6-month exercise regimen (data not shown).

Discussion

In the present study, the methylation of *ASC* was found to decrease in an age-dependent fashion and implied that ASC expression increased with age. Higher *ASC* methylation in the older exercise group than in the control group indicates that moderate exercise may restore *ASC* methylation to comparably younger levels. Neither moderate exercise nor age affected methylation of the *p15* tumor suppressor gene.

BMI and body fat were lower and VO_2max was higher in the young control group than in the older control group, therefore we analyzed the association of age and those clinical data with *ASC* methylation by using forced entry multivariable linear regression analysis following Pearson's correlation coefficient estimation, and found that age was the main factor associated with *ASC* methylation between young and older control groups. There was a difference in glucose values between the older control and older exercise groups, however, exercise was primarily associated with *ASC* methylation in those two groups.

Adequate amounts of cytokines are required for innate immune function, however, elevation of TNF-α, IL-1β, and/or IL-6 is observed in type 2 diabetes [21], rheumatoid arthritis [4] and atherosclerosis patients [2]. TNF-α, IL-6 levels [19] and IL-1, TNF-α levels [3, 6] have been reported to increase age-dependently as well. Thus, the development and progression of several age-related diseases are associated with chronic inflammation.

Chronic training has been shown to reduce the expression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [8, 9, 26] According to Greiwe et al. [10], expression of TNF-α expression in skeletal muscle is higher in elderly subjects compared to young subjects, but decreases after 3 months of resistance exercise. Our present results concerning *ASC* methylation are consistent with these studies. Since skeletal muscles are reported to produce and express cytokines regulated by exercise [20], *ASC* methylation should be measured not only in blood cells, but also in voluntary muscle. However, obtaining informed consent was quite difficult in volunteer subjects, which constitutes a challenge in future studies.

ASC is reported to be required for the induction not only of IL-1β, IL-18, but also of IL-6, IL-8, IL-10, and TNF through both caspase-1-dependent and -independent mechanisms [25]. Chronic moderate exercise reduces IL-6, TLR4 [23] and IL-6, IL-1β, TNF-α, and TLR4 expression [16]. Since TLR4 is one of the upstream receptors of ASC [14], ASC together with TLR4 might reduce inflammatory cytokines by chronic moderate exercise.

Our findings lack the measurement of cytokines, such as IL-1β and IL-6. Although the measurement of them is important, such studies with volunteer participants are usually complicated by difficulties in obtaining consent for larger amounts of blood. The same scenario applies to the measurement of ASC protein changes associated with *ASC* methylation. However, we have already been able to confirm that a decrease in ASC protein expression is associated with increased methylation [11, 29]. Also, the exercise period in this study was 6 months, but a one year regime may be more revealing of the effects of interval walking since other unknown factors may have affected ASC methylation in the current experiment.

Lastly, the *P15* gene was selected to investigate the influence of exercise on carcinogenesis. No influence was observed, however, *P15* became a methodologically good reference result. From this finding, discrepancies among groups in terms of season, classification, exercise quantity, and so on could be excluded.

As we described in Materials, the exercise group had just finished its 6-month exercise regimen when we collected blood from older subjects. Ideally, DNA samples from the exercise group should also have been collected prior to starting exercise, however, such DNA samples for methylation analysis were unobtainable at the time, since our experimental protocol was under consideration by the University's ethical committee. To strengthen the usefulness of our data, we are planning to compare the methylation data from a control group with the data after 6 months of exercise in a future study. Nonetheless, we believe that the present results are still useful as a novel finding which may impact the development of molecular research in sports medicine. As far as we have examined, the differences in methylation of the *ASC* gene were attributed to no other apparent factors besides training.

In summary, chronic moderate exercise, such as intense interval walking, can increase *ASC* methylation and thereby may suppress excess pro-inflammatory cytokine expression.

References

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Legend

Fig 1. Schematic view of the *ASC g*ene region analyzed in this study.

Seven CpG sites in the CpG island were designated for pyrosequencing analysis based on the effective region from Conway et al. (2000).

Fig 2. Methylation of *ASC* and age.

Difference in the mean methylation values of 7 sites between the young control group (n=34) and the older control group (n=153). Values are written as mean ± SE. ***p*<.01.

Fig 3. Methylation of *ASC* and exercise in older groups.

Left: Methylation rates of all 7 targeted sites. Open circles: older control group (n=153). Closed circles: older exercise group (n=230), after 6-month interval walking regimen.

Right: Difference in mean methylation values of the 7 sites between the older control group and the older exercise group. Values are written as mean ± SE. **p*<.05, ***p*<.01

Fig 4. Relationship between methylation of *ASC*, age, and exercise.

Dashed arrows indicate the schematically estimated age of youth restoration caused by the exercise. Values are written as mean ± SE. **p*<.05, ***p*<.01. Significance is versus older control group.

Table 1 Clinical data of subjects

	Young control group	Older control group	Older exercise group
Number (n)	34	153	230
Age range (years)	18-22	40-87	41-86
Age (years)	19.4±0.9	64.8 ± 7.5	66.2 ± 7.1
Height (cm)	165.3 ± 7.6	156.8 ± 7.4	155.8 ± 7.4
Weight	58.4 ± 11.3	58.9 ± 9.7	57.8 ± 8.9
BMI	21.1 ± 3.2**	24.0 ± 2.3	23.8 ± 1.9
Body Fat (%)	19.2 ± 8.0**	29.7 ± 6.5	30.7 ±6.7
HDL Cholesterol (mg/dl)	-	69.3 ± 15.9	69.0 ± 16.6
LDL Cholesterol (mg/dl)	-	131.9 ± 29.5	132.4 ± 28.3
Triglycerides (mg/dl)	-	108.2 ± 58.9	102.9 ± 49.4
VO_2max (ml/kg/min)	41.5 ±8.5**	23.8 ± 4.3	23.8 ± 5.1
Glucose (mg/dl)	-	101.9 ± 12.8	105.9 ± 19.9*

Data are written as mean ± SD. *p<.05, **p<.01 vs. Older control group

Table 2 Primers and conditions used for *ASC* and *p15* PCR

Gene	Forward primer (5'to 3')	Reverse primer (5'to 3')	Sequencing primer (5'to 3')	PCR conditions
ASC				
1st (423bp)	TGTATTAGTTGGTGTAAG TTTAGAGATAAGTAG	CACACCCACAACAAC TTCAACTTAA		95˚C-5m, (95˚C-30s; 50˚C-30s, 72 °C-30s) x3, (95 ˚C-30s, 58 °C-30s, 72 °C-30s)x47, 72 °C-5m
nested (237bp)	AGGGGATTAAGGGTG TAGTAAGGAA	Biotin -CTCCTCCACCAT CAAATTCTC	GGGATTTTGGAG TTATG	95˚C-5m, (95˚C-30s; 58˚C-30s, 72 °C-20s) x50, 72 °C-5m
P15 (133bp)	GTTTTTTTTTAGAAG TAATTTAGG	Biotin –CCTTCTACRACT TAAAACC	GTTAGGAAAAGTT	95˚C-5m, (95˚C-30s; 50˚C-30s, 72 °C-30s) x50, 72 °C-5m

Table 3 Association of data with *ASC* methylation was evaluated by forced entry multivariable linear regression analysis

	B	SE	Beta	p-Value
Age	0.042	0.012	-0.396	0.001
BMI (%)	0.005	0.052	0.009	0.920
Body fat (%)	0.043	0.023	-0.175	0.059
VO_2max (ml/kg/min)	0.012	0.030	-0.045	0.696

R^2=0.196, p<.001

Fig.1

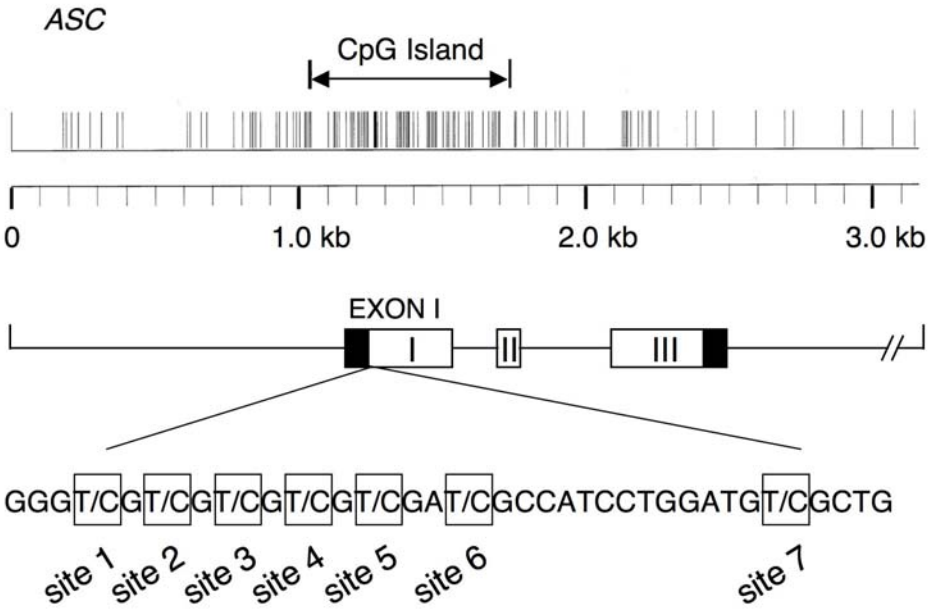

ASC
CpG Island
0
1.0 kb
2.0 kb
3.0 kb
EXON I
I
II
III
GGGT/CGT/CGT/CGT/CGT/CGAT/CGCCATCCTGGATGT/CGCTG
site 1
site 2
site 3
site 4
site 5
site 6
site 7

Fig.2

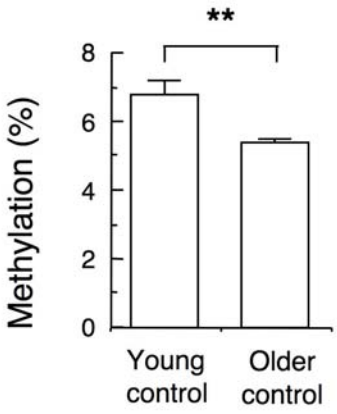
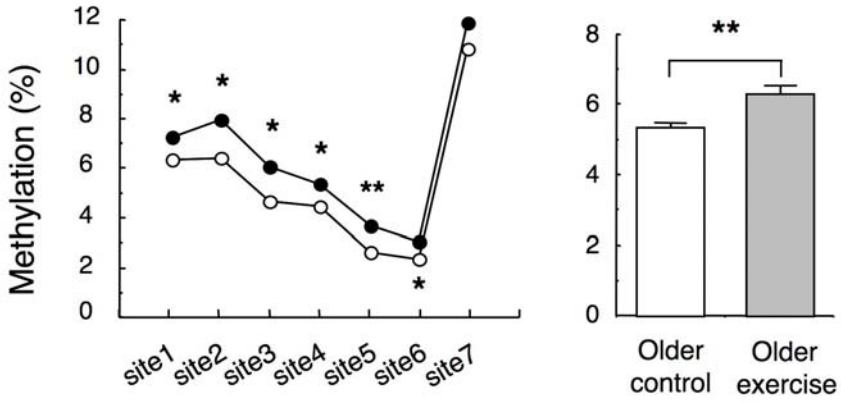


Fig.3


Methylation (%)
12
10
8
6
4
2
0
*
*
*
*
**
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site1
site2
site3
site4
site5
site6
site7
8
6
4
2
0
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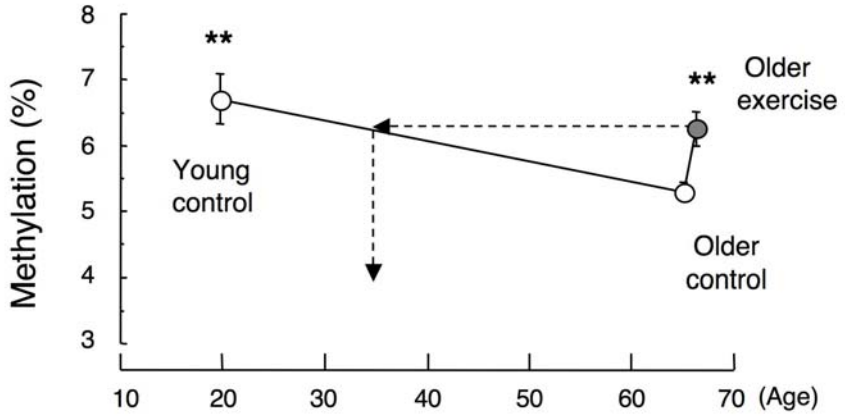
Older control
Older exercise

Fig.4


Methylation (%)
8
7
6
5
4
3
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Older
exercise
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10
20
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40
50
60
70
(Age)