Low Macrophage Accumulation in Skeletal Muscle of Obese Type 2 Diabetics and Elderly Subjects

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In addition to adipose tissue, recent studies suggest that skeletal muscle may also be a source of low-grade inflammation, particularly in inactive and/or overweight individuals. The aim of this study was to examine the presence of macrophages in skeletal muscle from obese subjects with type 2 diabetes (T2D) before and after a 9-month exercise program (vs. a non-exercising control group) (Study 1) and in young vs. elderly subjects (Study 2). In both studies, CD68+ macrophages in vastus lateralis biopsies were determined by immunohistochemistry and inflammation gene expression measured. Macrophage content (%) was calculated by the number of macrophages per 100 muscle fibers. In Study 1, we found relatively low numbers (2–3%) of CD68+ macrophages in skeletal muscle in obese T2D subjects (BMI = 37.3 ± 5.2 kg/m²), which were unchanged after a 9-month exercise program (P = 0.42). Similarly, in Study 2 (BMI = 27.1 ± 2.5 kg/m²), CD68+ macrophages were relatively low in muscle (4–5%) and were not different between young and elderly individuals (P = 0.42). However, elderly subjects had twofold higher CD68 and CD206 gene expression (both P < 0.002) than young participants. In both studies, CD68+ muscle macrophages were not associated with BMI. In conclusion, we found little evidence of macrophage accumulation in skeletal muscle in obese T2D subjects or in elderly individuals. A 9-month exercise program was not associated with a decrease in macrophage content.
to young subjects, elderly subjects would have lower muscle inflammation.

METHODS

Both studies were approved by the Pennington Biomedical Research Center Institutional Review Board and all participants gave written informed consent. Study 1 was an ancillary study to a randomized controlled trial in which 262 men and women with type 2 diabetes (T2D) and glycated hemoglobin (HbA1c) levels of 6.5% or higher were enrolled in one of four 9-month exercise programs (aerobic training, resistance training, combination of aerobic and resistance training, and a nonexercise control group) (10). A subset of 20 participants per group had skeletal muscle biopsies taken at baseline and post-intervention. Given that the overall finding of the parent study was that only the combination of aerobic and resistance training improved HbA1c levels compared to a nonexercise control group (10), we only examined skeletal muscle macrophages in participants that had both baseline and month 9 muscle biopsies in these two groups. This consisted of 7 non-exercisers (1 male, 6 females; age = 56.0 ± 5.5 years; BMI = 35.2 ± 4.4 kg/m²) vs. 7 subjects who underwent 9 months of aerobic and resistance training (1 male, 6 females; age = 54.8 ± 5.5 years; BMI = 39.4 ± 5.4 kg/m²). Study 2 consisted of 13 young (7 males, 6 females; age = 21–33 years; BMI = 25.2 ± 2.8 kg/m²) and 12 elderly (6 males, 6 females; age = 70–81 years; BMI = 26.9 ± 2.5 kg/m²) who were part of a cross-sectional study of age-associated alterations in skeletal muscle mitochondrial activity and had muscle biopsies taken (11). Elderly subjects (33.3 ± 9.2%) had significantly higher %body fat compared to young subjects (24.9 ± 7.9%, P = 0.03).

Immunohistochemistry

Macrophages were quantified with an antibody to CD68 (MO718; DAKO, Carpinteria, CA). To quantify macrophage number, three non-consecutive sections per sample were examined at ×20 magnification. The total area and the number of CD68+ macrophages and skeletal muscle fibers in the whole section were determined. Partial myofibers located on section borders were excluded as were CD68+ cells associated with the vasculature. Particular care was taken to exclude areas which did not contain muscle fibers (connective tissue or gaps due to sectioning artifacts) or had longitudinal fibers (sectioning artifacts), and to avoid areas where adipose tissue was present. Macrophage presence is expressed in two ways: macrophage content in the skeletal muscle biopsy (number of macrophages per number of cross-sectional fibers × 100%) and the number of macrophages per cross-sectional area of skeletal muscle. Data were analyzed using SPSS.V.19 (SPSS, Chicago, IL) and presented as mean ± s.d. or median (interquartile range).

Gene expression

Absolute quantification of messenger RNA (mRNA) expression was analyzed using ABI Prism 7900 (Applied Biosystems, Branchburg, NJ) and custom TaqMan gene expression measured against a standard curve for CD68 (Hs00154355_m1), CCL2 (Hs00234140_m1), CD40 (Hs00374176_m1), CD206 (Hs00267207_m1), CD11c (HS01015070_m1), and Arginase 1 (Hs00986897_m1). Samples were run in duplicate and expression levels normalized to RPLPO (Hs99999902_m1). For Study 2, we only had RNA from 10 young and 8 elderly subjects. Arginase 1 was not detected in 2/3 of the samples.

RESULTS

Muscle macrophage content and inflammation was unchanged after a 9-month exercise program in obese type 2 diabetics

In Study 1, controls had 2.2 (2.0–3.1%) skeletal muscle macrophages at baseline which was not different after 9 months (1.7 (0.9–6.95%); P = 0.85). Contrary to our hypothesis, subjects in the exercise group had 2.7 (2.0–3.2%) skeletal muscle macrophages at baseline which was unchanged after 9 months of the exercise program (4.0 (3.2–6.8%), P = 0.41) (Figure 1a,b). Similarly, inflammation gene expression levels were unchanged in either group (data not shown). Using a repeated measures ANOVA, there was no significant effect of time (P = 0.44) or group (P = 0.74) on %muscle macrophages, and no significant interaction between time and group (P = 0.90). Similar non-significant results were seen for inflammation gene expression (data not shown). There were no associations between baseline BMI and %macrophages and inflammation genes. Similar findings were seen when the number of macrophages per surface area was used in the above analyses (data not shown). Change in %macrophages between baseline and month 9 was not associated with changes in HbA1c, C-reactive protein, BMI, %fat, %fat-free mass or homeostasis model assessment (data not shown).

Higher CD68 and CD206 expression in elderly compared to young subjects, but no difference in macrophage content

In Study 2, elderly subjects had twofold higher CD68 (young = 0.4 ± 0.2; elderly = 0.8 ± 0.2 arbitrary unit (AU); P = 0.002) and CD206 mRNA levels (young = 0.4 ± 0.2; elderly = 0.9 ± 0.1 AU; P < 0.001) compared to young subjects. However, CD68+ macrophages were present in only 12 (6 young, 6 elderly) out of 25 skeletal muscle samples. In these 12 samples, %muscle macrophages were not different between young (4.7 (1.7–8.4%)) and elderly subjects (4.0 (0.6–6.1%); P = 0.42) (Figure 1c). Similar findings were seen when macrophages per cross-sectional area was analyzed (data not shown). There were no significant correlations between BMI or %fat and macrophage content (% or number of macrophages/area) or inflammation genes. There were no differences in CD11c, CCL2, and CD40 mRNA levels between young and old subjects (data not shown).

DISCUSSION

Recent studies in high-fat fed mouse models and humans have pointed to macrophage accumulation in obese skeletal muscle leading to the speculation that these macrophages may secrete additional proinflammatory mediators that contribute to the chronic-low grade inflammatory state seen in obesity (1,2,4,12). In contrast to these previous findings, our results show relatively low amounts (2–3%) of macrophages in skeletal muscle of obese type 2 diabetics, which were unchanged following a 9-month exercise program. In support of this, there were no significant changes in inflammation gene expression with exercise training. Varma et al. previously reported 2.5-fold higher CD68+ muscle macrophages in eight obese (25%) compared to eight lean (10%) individuals (1). Subjects in Varma's study and our study had similar BMIs (Tam = 37.3 kg/m²; Varma = 36.9 kg/m²), and similar immunohistochemistry methods were used. Another study in subjects with impaired glucose tolerance (BMI = 24.9 kg/m²) also showed increased CD68+ skeletal muscle macrophages and CD68, IL-6, tumor necrosis factor-α (TNF-α), and toll-like receptor-4 (TLR-4) protein levels; however, that study did not describe the quantification of macrophages or report numbers of macrophages...
in the skeletal muscle (4). It may be considered a limitation of our study that we did not quantify macrophages in a lean control group; however, the %macrophages (2–3%) in our study are considerably lower than both the lean (10%) and obese groups (25%) in Varma et al.’s study. It is possible that cross-contamination of adipose tissue in skeletal muscle leads to artificially higher macrophage numbers or inflammation levels, as was seen in some skeletal muscle samples from our study (Figure 1d). In agreement, the first study to report macrophages in skeletal muscle only showed them in the intermuscular adipose tissue region, and two other studies in morbidly obese subjects (BMI range = 37–45 kg/m²) reported low CD68 and MCP1 mRNA levels in skeletal muscle (6,7). Nevertheless, it is well-demonstrated in vitro that an inflammatory response in muscle cells can lead to impaired insulin signaling (1,4,5,12). Further studies investigating macrophage accumulation and the association with lipid deposition in skeletal muscle in vitro and in vivo are necessary to explore the presence of inflammation in skeletal muscle in obesity and T2D.

Our study also found twofold higher CD68 and CD206 mRNA expression in elderly compared to young subjects. Despite this, skeletal muscle macrophages were not different between the two groups (young = 4.7%, elderly = 4.0%). Aging has been suggested to be a state of low-grade inflammation (8) and elderly people have two to threefold higher circulating IL-6 (13). To our knowledge, the only study that has examined skeletal muscle macrophages in young and elderly patients showed that CD68+ macrophages are lower in vastus lateralis from elderly subjects (9). Notably, regardless of age, we were unable to demonstrate higher macrophage accumulation in skeletal muscle of mostly overweight subjects as has been reported in other studies (1).

In conclusion, we found little evidence of macrophage accumulation in skeletal muscle in obese type 2 diabetic and elderly subjects. Furthermore, macrophages in skeletal muscle were not influenced by 9 months of combined aerobic and resistance exercise despite an improvement in glucose control. Future studies need to carefully examine the presence of adipose tissue in skeletal muscle depots as contamination with adipose tissue may potentially lead to artificially higher numbers of skeletal muscle macrophages being reported.

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DISCLOSURE
The authors declared no conflict of interest.

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