Exercising alters the IGF axis in vivo and increases p53 protein in prostate tumor cells in vitro

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Submitted 18 August 2003; accepted in final form 10 September 2003

Exercise alters the IGF axis in vivo and increases p53 protein in prostate tumor cells in vitro. J Appl Physiol 96: 450–454, 2004; 10.1152/japplphysiol.00871.2003.—Epidemiological studies report that regular physical activity can reduce the risk for prostate cancer, the most common solid-tumor cancer in US men. Regular exercise alters the serum IGF axis in vivo and reduces cell proliferation while increasing apoptosis in serum-stimulated LNCaP prostate cancer cells in vitro. The present study tests the hypothesis that these effects on tumor cell lines are mediated by enhancement of the function of the p53 gene known to arrest cell growth and induce apoptosis. When LNCaP cells were cultured in exercise serum and compared with control serum, cell growth was reduced by 27%, and there was a similar 33% decrease in proliferating cell nuclear antigen protein, a marker for cell cycling. Apoptosis was increased by 371% with the exercise serum, and there was a 100% increase in p53 protein (7.52 ± 2.0 vs. 38.2 ± 2.0 pg/μg protein). When serum was used to stimulate LN-56 cells, a cell line with nonfunctional p53 derived from LNCaP, no significant reduction in cell growth or increase in apoptosis with the exercise serum was observed. These results indicate that exercise training alters serum factors in vivo that increase cellular p53 protein content and is associated with reduced growth and induced apoptosis in LNCaP prostate cancer cells in vitro.

LNCaP cells; LN-56 cells; cell growth; apoptosis; proliferating cell nuclear antigen

Prostate cancer is the most common solid-tumor cancer in US men and is a major cause of cancer mortality (17). The international variation in prostate cancer mortality as well as migration studies indicate that environmental factors play a key role in the very high incidence of this cancer in the US (12, 30, 36). Two environmental factors that have been implicated are a typical Western high-fat diet and physical inactivity. In a recent review of the literature on physical activity and the risk for prostate cancer, Thune and Furberg (37) found that 14 of 28 epidemiological studies reported that increased occupational or leisure time activity reduced the risk for prostate cancer by 10–70%.

Previous studies have demonstrated that a low-fat diet and/or regular physical activity results in changes in serum in vivo that reduce the growth of serum-stimulated LNCaP and LAPC-4 prostate tumor cell lines in vitro (23, 38, 39). More important than the reduction in the rate of tumor cell growth was the fact that a significant number of the tumor cells showed signs of apoptosis. It is well recognized that the induction of apoptosis is important for determining the development of clinical cancer (11). This fact may explain why clinical prostate cancer is rare in East Asian men, even though, at autopsy, 20% have isolated malignancy in the prostate (4). When Asian men migrate to the US and adopt our lifestyle, clinical prostate cancer and mortality from prostate cancer approaches US levels (31). Some of the serum changes resulting from US men adopting a low-fat diet and/or regular exercise that might affect the growth and apoptosis of tumor cells include reductions in insulin, estradiol, free testosterone, and IGF-I, as well as increases in sex hormone-binding globulin (SHBG) and IGF binding protein-1 (IGFBP-1) (22, 23, 39). Of these factors, the reduction in serum IGF-I appears to be the most important. When insulin, estradiol, and testosterone were added to the serum from men following a low-fat diet and exercise, they accounted for only one-half of the reduction in tumor cell growth (39). Thus we shifted focus to the IGF axis, which is composed of IGF-I, IGF-II, IGF receptors, and six different IGFBPs (7). When IGF-I was added to the serum of men following a low-fat diet and/or exercise, the reduction in tumor cell growth was completely eliminated (1, 22).

This study focuses on the ability of IGF-I to suppress the normal function of the p53 gene. The specific biological roles attributed to p53 are very complex, but p53 essentially protects the genome from mutations and/or genetic alternations. When defects are found in DNA, the p53 protein is stabilized, which activates other downstream genes or factors to cause cell cycle arrest or DNA repair or to induce apoptosis (11, 42). IGF-I has been documented to suppress these actions of p53 (11, 14, 20, 25, 35). As we have documented that a low-fat diet and/or exercise decreases serum levels of IGF-I while increasing serum IGFBP-1 to further reduce free IGF-I, we hypothesized that these serum changes may increase tumor p53 protein content, reduce serum-stimulated tumor cell growth, and initiate apoptosis. To test this hypothesis, we have measured the effect of exercise on serum-stimulated p53 protein content in the LNCaP prostate cell line. Furthermore, we have tested the effects of exercise on serum-stimulated growth and apoptosis of LN-56 cells, a line with nonfunctional p53 derived from LNCaP cells. The results from both of these approaches indicate that serum from men who regularly exercise does increase...
the p53 protein in LNCaP tumor cells and that this is likely important for the reduced cell growth and the induction of apoptosis.

MATERIALS AND METHODS

Subjects. Similar aged (62 ± 2 and 60 ± 3 yr, respectively) groups of control (n = 10) and exercise (n = 12) volunteer men were studied. The control group consisted of men who were considered to be at risk for prostate cancer due to their sedentary lifestyle and dietary habits, but who had prostate-specific antigen values in the normal range. The exercise group attended the Adult Fitness Program at the University of Nevada Las Vegas. Volunteers were requested who had participated in the program for at least 10 yr; the average was 14.7 yr. The program was held 5 days/wk for 1 h and consisted of warm-up and flexibility exercises including calisthenics and swimming laps in the pool, as described previously (9). There was no dietary intervention in the University of Nevada Las Vegas Adult Fitness Program. Fasting blood samples were obtained from the subjects in the morning, allowed to clot, and were centrifuged to obtain serum. The serum was frozen and stored at −80°C until used for analyses. Serum samples were analyzed for insulin, IGF-I, and IGFBP-1 by using ELISA kits from Diagnostic Systems (Webster, TX) and reported in an earlier paper (1). The University of California Los Angeles Institutional Review Board approved the study, and informed consent was obtained from the subjects.

Cell culture. Androgen-dependent LNCaP prostate tumor cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in 75-cm² flasks (Falcon Primaria, Bedford, MA) in RPMI-1640 medium without phenol red and supplemented with 10% FBS, 200 IU penicillin, 200 mg/ml streptomycin, and 4 nM t-glutamine (Omega Scientific, Tarzana, CA). The cultures were maintained at 37°C and supplemented with 5% CO₂ in a humidified incubator. Cells were passaged routinely at 80% confluence, and fresh medium was replaced every third day. Cells used in experiments were not passaged >10 times.

To assess the potential role of p53 in exercise-stimulated LNCaP cancer cell growth reduction and induced apoptosis, we studied an LNCaP-derived cell line, LN-56, in which p53 was rendered nonfunctional by expression of a dominant negative fragment of p53, known as genetic suppressor element 56 (24, 29). The LN-56 was a generous gift from Dr. Nancy L. Weigel (Baylor College of Medicine, Houston, TX). LN-56 cells were grown in RPMI medium (Omega Scientific) containing 10% FBS (Omega Scientific), 10 mM HEPES (Omega Scientific), 1 mM sodium pyruvate (Omega Scientific), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 2 mM t-glutamine (Omega Scientific), and 400 μg/ml G418 (Omega Scientific). The cultures were maintained at 37°C and supplemented with 5% CO₂ in a humidified incubator. Cells were passaged routinely at 80% confluence, and fresh medium was replaced every third day.

Growth assay. Cells in the Falcon flasks were detached with 0.25% Trypsin-EDTA solution (Sigma Chemical), centrifuged at 3,000 g for 5 min at 10°C, and resuspended in fresh medium. Cell viability was assessed via Trypan blue exclusion (23, 39). Experiments were carried out in 96-well tissue culture plates (Falcon, Primaria). Cells were plated (5 × 10^4 cells/well) in 96-well plates and allowed to attach and stabilize for 24 h. After the 24-h stabilization period, fresh medium (RPMI-1640 medium, 200 IU penicillin, 200 mg/ml streptomycin, and 4 nM t-glutamine) with 10% FBS or 10% human serum was added to the wells in triplicates. The plates were then incubated (37°C, 5% CO₂) for 48 h. Cell growth was determined by CellTiter 96 AQueous assay (Promega, Madison, WI). This method has been shown to correlate (<5% difference) with ³Hthymidine incorporation (34) and with other proliferation assays, such as MTT and XTT (27). Using manual counting, our laboratory has reported a correlation of r = 0.98 for the CellTiter assay (23).

Apoptosis assay. Experiments were carried out in 96-well tissue culture plates at a density of 10 × 10^3 cells/well. Cells were prepared and plated for 24 h as mentioned above. Apoptosis was measured at the end of the 48-h incubation period with FBS, control, or exercise sera. The levels of apoptosis were assessed by Cell Death Detection ELISA Plus (Roche Applied Science, Indianapolis, IN). This apoptosis assay is based on a quantitative sandwich enzyme immunoassay principle by using mouse monoclonal antibodies directed against DNA and histones and allows the specific determination of mono- and oligonucleosomes in the cytoplasm fraction of lysates. Background using incubation buffer and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) solution was subtracted from the absorbance measurements (405–490 nm). The results are expressed in micromolars of mono- and oligonucleosomes. In previous studies with the same serum samples, we have used terminal deoxynucleotidyl transferase-mediated dUTP and annexin V assays to determine apoptosis and found results similar to those now reported for the Cell Death Detection ELISA (1).

Proliferating cell nuclear antigen and p53 assays. LNCaP cells were initially plated at a density of 150,000 cells/10-cm dish and allowed to attach and stabilize for 24 h. After the 24-h stabilization period, fresh medium (RPMI-1640 medium, 200 IU penicillin, 200 mg/ml streptomycin, and 4 nM t-glutamine) with 10% FBS or 10% human serum was added to the dishes. Cells were then incubated for 48 h. After the incubation period, cells were washed once in PBS and were lysed with 5× passive lysis buffer containing protease inhibitors (Promega). After 30-min incubation at room temperature, lysates were centrifuged for 10 min at 3,000 g, and the supernatants were collected. A Bradford protein assay (Bio-Rad Laboratory, Richmond, CA) was used to determine total protein concentration in lysates, and cell suspension buffer was used to adjust the protein concentrations to 10 μg/ml.

Proliferating cell nuclear antigen (PCNA) protein concentration in cell lysate supernatants was measured by PCNA ELISA kit (Oncogene Research Products, San Diego, CA). This assay also correlates with bromodeoxyuridine cell proliferation assay. p53 ELISA kit (Oncogene Research Products) was used to measure p53 protein concentration in cell lysate supernatants, according to the manufacturer’s protocol.

Statistical analysis. All assays for both the control and exercise samples were done on the same 96-well plates, and the results are expressed as a percentage of the control results. Statistical analysis (InStat Statistical Software, Graphpad Prism, San Diego, CA) was performed by Student’s t-test for two variable data sets. P < 0.05 was considered significant. Data are expressed as means ± SE.

RESULTS

Table 1 shows the body mass index (BMI) data and the results for serum analyses of insulin, IGF-I, and IGFBP-1.

Table 1. Comparison of body mass index and serum IGF axis for control and exercise subjects

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exercise</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>BMI</td>
<td>31.5 ± 1.6</td>
<td>26.5 ± 1</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>110 ± 38</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>IGF-1, ng/ml</td>
<td>336 ± 41</td>
<td>128 ± 12</td>
</tr>
<tr>
<td>IGFBP-1, ng/ml</td>
<td>22 ± 6</td>
<td>42 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. Serum concentrations reflect fasting levels. BMI, body mass index; IGFBP-1, IGF binding protein-1. All exercise values were significantly different from control, P < 0.05. These data were previously published (1).
BMI, insulin, and IGF-I were significantly lower in the exercise subjects, whereas IGFBP-1 was significantly higher. These data have all been reported previously (1).

Figure 1 shows the results for the serum-stimulated growth assays. For the LNCaP cells, growth with the exercise serum was reduced by 27% compared with the control. When the human serum was used to stimulate the LN-56 cells, there was no significant difference between the two groups, with the exercise serum-stimulated growth being 91% of control.

Figure 2 shows the results from the PCNA protein ELISA in LNCaP cells after 2 days of serum stimulation. PCNA, a marker for cell cycling, was reduced by 33% (9.0 ± 0.16 vs. 13.6 ± 0.36 PCNA units/μg protein) with the exercise serum. These data show good agreement with the 27% reduction in cell growth in Fig. 1 for the CellTiter assay.

Figure 3 shows the results from the apoptosis assays. For the LNCaP cells, the exercise serum increased apoptosis by 371% (3.3 ± 0.1 vs. 38.2 ± 2.0 pg/μg protein) compared with control. For the LN-56 cells, just the opposite was observed as apoptosis was reduced by 50% (0.35 ± 0.02 vs. 0.7 ± 0.07 μU) compared with control.

Figure 4 shows the results from the p53 protein ELISA in LNCaP cells after 2 days of serum stimulation. The p53 protein increased by 100% (7.5 ± 2.0 vs. 38.2 ± 2.0 pg/μg protein) in the exercise serum-stimulated cells.

DISCUSSION

The results from this study confirm our earlier report (1) showing that serum obtained from men (62 ± 2 yr old) after a regular exercise program for >10 yr reduced the growth rate and induced apoptosis of the LNCaP prostate cancer cell line. The reduction in cell growth over 48 h was 27% compared with serum from sedentary control men and was in agreement with the 33% reduction in PCNA protein, a marker for cell cycling. As we hypothesized, the reduction in cell growth and the induction of apoptosis were associated with a 100% increase in p53 protein. According to Gurumurthy et al. (11), most prostate cancer cells have intact cell death programs, but they fail to initiate or execute them. The results of this study indicate that regular exercise alters serum factors that allow the prostate cancer cells to initiate apoptosis, as opposed to control serum in which very little apoptosis was observed in the present and previous studies (1, 23). Changes in the IGF axis, including reductions in IGF-I and increases in IGFBP-1, appear to be the most significant factors, as our laboratory previously reported that changes in sex steroid hormones were found to be less important (40).

Regular exercise has an acute, insulin-like effect and is well known to enhance insulin sensitivity (10). Thus it is not surprising that the exercise subjects had lower fasting insulin levels compared with the sedentary controls. Elevated serum insulin has been reported to be a risk factor for prostate cancer (15, 16). The observed lower serum insulin in the exercise group could impact directly on the cancer cells to reduce growth. However, we previously reported that this is a minor
factor in our results (39). More important than the direct effect of insulin on the tumor cells is the impact the lower serum level of insulin has on the liver. Insulin stimulates the liver to produce IGF-I as well as inhibits the production of IGFBP-1 and SHBG (26, 27, 32). The reduction in SHBG would in-
crease free testosterone, as we previously reported (40). This could be significant, as all early-stage prostate cancer is andro-
gen dependent. However, our laboratory’s previous study indi-
cates that this is also a minor factor in the observed reduction in LNCaP cancer cell growth (39).

The reduction in serum IGF-I and increase in IGFBP-1 appear to be the most significant factors. In our laboratory’s previous study (23), we showed that serum IGF-I was directly correlated with tumor cell growth, whereas IGFBP-1 was inversely related to tumor cell growth. Furthermore, when IGF-I was added to the serum from the diet and/or exercise group, the reduction in LNCaP growth was eliminated (1, 23). IGF-I has been reported to be a biomarker to predict prostate cancer in prospective studies (5, 13, 33) and has also been reported to be a risk factor in case-control studies (6, 21, 43). In addition, one of the case-control studies reported that ele-

vated IGFBP-1 reduced the risk for prostate cancer (6). IGF-I has been shown in many studies to be a suppressor of apoptosis (11, 14, 25). Recently, Heron-Milhavet and LeRoith (14) reported that IGF-I suppressed apoptosis in ultraviolet-dam-
aged cells via activation of the p38 mitogen-activated protein kinase pathway. The suppression of apoptosis by IGF-I was associated with a decrease in cellular p53 protein content with no change in p53 mRNA. The decrease in p53 protein was associated with an increase in murine double minute 2 protein and mRNA. Murine double minute 2 is a RING (really interesting new gene) finger ubiquitin ligase protein that binds to p53 and shuttles it to the cytoplasm for degradation. Our results showed that serum from exercise subjects with very low levels of IGF-I was associated with higher p53 protein and with a significantly higher amount of apoptosis in LNCaP cells sug-

gest the possible involvement of the p38 pathway. The results from the LN-56 cells further suggest this conclusion, as exercise serum had no effect on cell growth or apoptosis. In fact, apoptosis was reduced in all 12 of the exercise serum-stimu-
lated LN-56 cells compared with control. The reason for this is unknown and may be due to a lack of sensitivity at very low levels. This was opposite to the response in wild-type LNCaP cells. The increase in p53 protein in the exercise serum-stimulated LNCaP cells was associated with reduced growth and reduced PCNA protein. This response could be the result of p53 increasing p21, growth arrest and DNA damage induc-
uble proteins, and/or other gene proteins that reduce cyclin kinase activity and cell cycling (41). The increase in apoptosis in the exercise serum-stimulated LNCaP cell could be the result of the increased p53 protein increasing the ratio of Bax to Bcl-2 and activation of caspases known to induce apoptosis (18). These possible downstream effects from elevated levels of p53 protein in the tumor cells remain to be confirmed. A limitation to this study is the fact that it was not random-
ized. In addition, there were many differences between the two groups, as previously reported (1). However, the lower serum levels of triglycerides and insulin are well-documented re-
sponses to regular exercise, as is a lower BMI. That the lower BMI itself is not the factor responsible for the lower insulin and decrease in LNCaP cell growth and increase in apoptosis is suggested by our laboratory’s previous studies reporting the observed changes in serum insulin and in tumor cell growth and apoptosis after just 11 days of diet and exercise while the men remained obese (BMI > 30) (23, 38). In the Swedish Twin Study, Jonsson et al. (19) found no clear association between BMI and prostate cancer. Furthermore, Evenson et al. (8) reported that the hazard ratio (HR) for cancer mortality in men showed a significant reduction for fitness levels, with the highest quintile of fitness having an HR of 0.47. When they adjusted for BMI, there was no meaningful change in the HR, suggesting that fitness was more important than BMI. Our laboratory has also reported in several papers that 2–3 wk of diet and exercise intervention in obese men or women signifi-
cantly lowers serum insulin while the subjects remain obese (3, 23, 39, 40). Furthermore, the changes in body weight did not correlate with reductions in serum insulin. In our animal model, we have shown that insulin resistance precedes any changes in fat cell size or body fat content when animals are placed on high-fat, refined-sugar diet (2).

In summary, the results from this study show that regular, moderate-to-intensive exercise in older men alters the IGF axis in vivo, reducing serum IGF-I, while increasing IGFBP-1. These changes in serum affect LNCaP prostate cancer cells in vitro to increase p53 protein, reduce cell growth, and increase apoptosis. These results provide a mechanism explaining the epidemiological data reporting a reduction in prostate cancer risk in men who are physically active.

REFERENCES