Metformin reduces the stimulatory effect of obesity on in vivo Walker-256 tumor development and increases the area of tumor necrosis

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A B S T R A C T

Aims: The objective of this study was to analyze the influence of obesity and insulin resistance on tumor development and, in turn, the effect of insulin sensitizing agents.

Main methods: Male offspring of Wistar rats received monosodium glutamate (400 mg/kg) (obese) or saline (control) from the second to sixth day after birth. Sixteen-week-old control and obese rats received 5 × 10⁶ Walker-256 tumor cells, subcutaneously injected into the right flank. Some of the obese and control rats received concomitant treatment with metformin (300 mg/kg) by gavage. At the 18th week, obesity was characterized. The percentage of rats that developed tumors, the tumor relative weight and the percentage of cachexia incidence were analyzed. The tumor tissue was evaluated histologically by means of hematoxylin and eosin staining.

Key findings: Metformin did not correct the insulin resistance in obese rats. The tumor development was significantly higher in the obese group, whereas metformin treatment reduced it. After pathological analysis, we observed that the tumor tissues were similar in all groups except for adipocytes, which were found in greater quantity in the obese and metformin-treated obese groups. The area of tumor necrosis was higher in the group treated with metformin when compared with the untreated one. The reduction occurred independently of the correction of insulin resistance. Metformin increased the area of necrosis in tumor tissues, which may have contributed to the reduced tumor development.

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Introduction

The prevalence of excess weight and obesity in most developed countries has been increasing markedly over the past two decades, and the association between obesity and other chronic diseases is also increasing (Hill 2006; Zimmet et al., 2001).

Epidemiological studies have associated obesity with a wide variety of cancers (Calle and Kaaks, 2004; Giovannucci and Michaud, 2007; Hursting et al., 2007; Jee et al., 2005). The insulin resistance, hyperinsulinemia, oxidative stress and chronic inflammation often present in obesity can be the mechanisms by which obesity induces or promotes tumorigenesis (Hursting et al., 2007). In fact, chronic hyperinsulinemia has been associated with cancers of the colon, breast, pancreas, and endometrium (Calle and Kaaks, 2004; Giovannucci and Michaud, 2007). These tumorigenic effects of insulin may be directly mediated by insulin receptors in the (pre)neoplastic cells, or they might be due to related changes in endogenous hormone metabolism (Calle and Kaaks, 2004). Insulin promotes the synthesis and biological activity of insulin-like growth factor 1 (IGF-1), and both can stimulate cell proliferation contributing to greater tumor development in obesity (Calle and Kaaks, 2004; Pollak, 2007).

Metformin, an antidiabetic drug, can have a direct antitumoral effect or can act indirectly to improve insulin sensitivity (Evans et al., 2007; Pollak, 2007; Schneider et al., 2001). Metformin lowers the elevated insulin levels found in type 2 diabetes by inhibiting glucose uptake by muscle and adipose tissue by activation of AMP-activated protein kinase (AMPK) and can activate the same LKB1/AMPK pathway in cancer cells in vitro (Algire et al., 2008; Ropelle et al., 2007; Zakikhani et al., 2006). In these cells, the consequences include reduced signaling at mTOR and growth inhibition (Algire et al., 2008; Zakikhani et al., 2006). Thus, although it is already known that obesity increases the risk of developing cancer and that metformin may have an antitumor effect in this condition, further studies...
are needed to better assess the association between obesity and cancer and the effect of metformin on it. Therefore, we investigated the influence of experimental obesity on tumor development and the effect of in vivo metformin treatment to better understand the relationship of tumor development and obesity and the influence on it of insulin-sensitizing agents. For this we used the MSG model of obesity in rats and Walker-256 tumor cells. We analyzed tumor development, the cachexia incidence and the effect of metformin on these parameters.

Materials and methods

Animals and induction of obesity

Male offspring of Wistar rats from our own breeding colony received daily subcutaneous injections of monosodium glutamate—MSG (400 mg/kg body weight; Sigma-Aldrich, Germany) dissolved in 0.9% NaCl from the second to sixth days after birth. Control littersmates received an equivalent volume of the vehicle (Fig. 1). The rats were weaned on the 21st day and were kept in standard cages under controlled light (12-h light/dark phase) and temperature conditions (22 ± 1 °C), with free access to food and water.

The experimental procedures were approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences, University of São Paulo, Brazil (Protocol n°007/04).

Tumor xenograft model and metformin treatment

In 16-week-old control (C) and obese-MSG (O) rats, 5 × 10^5 Walker-256 tumor cells were inoculated s.c. in the right flank. These rats were randomized to four groups: control tumor (CT), control tumor metformin (CTM), obese-MSG tumor (OT) and obese-MSG tumor metformin (OTM). The treatment with metformin (Glifage, Merck, Brazil) started on the same day that tumor cells were inoculated. The CTM and OTM rats received a dose of 300 mg/kg body weight of metformin by gavage for 15 days (Fig. 1).

Characterization of obesity and analysis of metabolic parameters

On the day of the experiment, after 5 h of food deprivation, the rats in all the groups were weighed and blood samples were taken from the descending aorta under sodium thiopental anesthesia (50 mg/kg, intraperitoneally, Cristália, Brazil). The blood samples were kept at room temperature for half an hour to coagulate, followed by centrifugation at 1900×g and 20 °C. The serum isolated was frozen at −80 °C for assessment of biochemical parameters. Glucose levels and the lipid profile were assessed spectrophotometrically using specific commercial kits (colorimetric method, Celm, Brazil). Insulin concentration was determined using a rat-specific radioimmunoassay kit (Linco, USA). The Lee index was calculated as follows: weight body weight^1/3 (g)/nasal-anal length (cm) × 100. The white adipose tissue (periepi-
dymidymal and retroperitoneal) and the lean mass (soleus, extensor digitorum longus (EDL) and gastrocnemius muscle) were weighed. The lipid peroxidation rate was determined using the thiobarbituric acid reactive substance (TBARS) assay. To assess the serum insulin levels, it was necessary to add two groups: control without tumor and obese-MSG without tumor.

Intravenous insulin tolerance test (ITT)

Tail blood samples were collected before and 4, 8, 12 and 16 min after an intravenous injection of regular insulin (0.75 U/kg bw, Biobras, Brazil). The constant rate for blood glucose disappearance during the test (kITT) was calculated based on the linear regression of the neperian logarithm of glucose concentrations. Blood glucose concentration was measured using a Glucometer and glucose strips (Advantage®, Roche, Brazil).

Intravenous glucose tolerance test (GTT)

This test was performed at noon on the 18th week of age of the animals. Tail blood samples were collected before and 5, 10, 20, 30 and 60 min after an intravenous injection of glucose (0.75 g/kg bw). Blood glucose concentration was measured using a Glucometer and glucose strips (Advantage®, Roche, Brazil).

Analysis of food intake

To assess food intake, it was necessary to add two groups: control without tumor and obese-MSG without tumor. The food intake was calculated and represented as the average food intake for 3 consecutive days at the 18th week of age of animals.

Tumor evaluation

At the 18th week of age, tumor weight (g/100 g body weight) and tumor volume (cm^3/100 g body weight) were evaluated. Tumor volume was calculated using the formula (0.5 × a × (b^2)) (where a is the larger diameter and b is the smaller diameter) with an electronic caliper as described previously (Kato et al., 1994). The percentage of rats that were inoculated with and developed the tumor was also calculated.

Histological analysis

After fixation with paraformaldehyde (4%) for 24 h, the tumor tissues were subjected to processing, during which they were dehydrated with increasing concentrations of alcohol (alcohol 70, 85, 95 and 100%), diaphanized, bathed in xylene and embedded in Paraplast (Paraplast X-tra, SEM). They were then cut into sections with a rotary microtome. The 6 μm thick sections were mounted on slides and stained with hematoxylin–eosin (H & E).

The slides were analyzed with a optical microscope (AXIOSKOP Zeiss, Germany) using a 10× and 20× objective for the overall assessment of tumor tissue. They were then photographed using an analog camera (Samsung, SHC-410 NAD, Korea). Quantitative analysis of slides was performed using the program ImageJ.
The pathological analysis of the tumor tissues for the presence of viable tumor cells (proliferating cells), cells in necrosis or apoptosis, adipocytes and blood vessels was performed.

Cachexia incidence

Loss of body mass (LBM) was calculated as follows:

\[ \text{LBM} = 100 \times \frac{(\text{BMi} - \text{BMf} + \text{MT} + \text{GBM})}{(\text{BMi} + \text{GBM})}, \]

where BMi: initial body mass of a rat with tumor (g); BMf: final body mass of a rat with tumor (g); MT: tumor mass (g); and GBM: body mass gain of a rat without tumor during the 15 days of the experiment (g).

The rats were considered cachectic when the LBM was higher than 10%. The cachexia incidence corresponds to the percentage of rats that became cachectic.

Data analysis

Data are given as mean ± SEM and n denotes the number of animals used. Data were analyzed using the Student’s t-test for comparison of two groups or one-way ANOVA followed by Tukey’s post-hoc test for multiple comparisons. P values less than 0.05 were considered significant.

Results

Characterization of obesity and analysis of metabolic parameters

The Lee index and the periepididymal and retroperitoneal adipose tissue weights were higher in the OT group than in the CT group. The lean mass (soleus and EDL muscle) weight of the OT group was not significantly different from that of CT, but the gastrocnemius muscle was significantly smaller in the OT group. In addition, serum triglycerides and very low density lipoprotein (VLDL) cholesterol, but not total cholesterol levels, were higher in the OT group than in the CT group. Lipid peroxidation rate was significantly higher in the OT group. Serum glucose levels were similar in all groups (Table 1).

Metformin did correct the dyslipidemia and reduced the periepididymal and retroperitoneal adipose tissue weight as well as the lipid peroxidation rate. Weight loss was significantly higher in the OT group than in the CT group (Table 1), but metformin did not correct this parameter.

Obese rats without tumors had higher serum levels of insulin than obese rats with tumors. The presence of a tumor reduced insulin levels in both CT and OT groups. Metformin did not alter the already reduced insulin levels in these latter groups (Fig. 2).

The constant rate for blood glucose disappearance during the kITT test obtained after insulin overload was significantly smaller in the OT rats when compared to the CT rats, indicating insulin resistance in these rats. Metformin did not correct this parameter (Fig. 3A and B).

The basal glucose levels (T0) (before the i.v. injection of 0.75 mg/kg glucose) was not different between groups. The peak glucose occurred 5 min after the glucose load, with a decline in levels after this time (T5). The area under the glucose concentration curve in blood was significantly higher in the OT group, showing glucose intolerance. Metformin did not correct this parameter (Fig. 3C and D).

Analysis of food intake

The food intake was significantly lower in the groups with tumor (CT, CTM, OT and OTM) when compared with their respective groups without tumor. Metformin reduced the food intake only in the OTM group (Fig. 4).

Analysis of tumor development and percentage of rats that developed tumors

Tumor development and the percentage of rats that developed tumors were significantly higher in the OT group when compared with the CT group. Both parameters were reduced by metformin treatment (Fig. 5). After pathological analysis, we observed that the tumor tissues were similar in all groups except for adipocytes, which were found in greater quantity in the OT and OTM groups (Fig. 6). The area of tumor necrosis was higher in groups treated with metformin when compared with the other groups (CT 33.1 vs CTM 52.6, OT 34.2 vs OTM 58.7, n = 10, *p < 0.05).

Cachexia incidence

The cachexia incidence was higher in the OT group than in the other groups; metformin did not correct this parameter (OT 90*** vs. CT 50, CTM 50, OTM 100%, n = 18, ***p < 0.0001).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Biological parameters of the animals.</th>
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<tbody>
<tr>
<td></td>
<td>CT</td>
</tr>
<tr>
<td>Initial weight (g)</td>
<td>386.2 ± 7.2</td>
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<tr>
<td>Final weight (g)</td>
<td>384.0 ± 9.5</td>
</tr>
<tr>
<td>Carcass weight (g)</td>
<td>362.5 ± 9.8</td>
</tr>
<tr>
<td>Weight loss (g)</td>
<td>22.2 ± 3.8</td>
</tr>
<tr>
<td>Lee index (&gt;100)</td>
<td>29.6 ± 0.2</td>
</tr>
<tr>
<td>Periepididymal adipose tissue (g/100 g bw)</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Retroperitoneal adipose tissue (g/100 g bw)</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Soleus muscle (g/100 g bw)</td>
<td>0.041 ± 0.002</td>
</tr>
<tr>
<td>EDL muscle (g/100 g bw)</td>
<td>0.041 ± 0.001</td>
</tr>
<tr>
<td>Gastrocnemius muscle</td>
<td>0.509 ± 0.012</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>94.2 ± 6.2</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>48.6 ± 3.5</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>109.5 ± 22.7</td>
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<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>58.0 ± 0.5</td>
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<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>27.8 ± 3.8</td>
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<tr>
<td>VLDL-cholesterol (mg/dL)</td>
<td>23.8 ± 4.7</td>
</tr>
<tr>
<td>Serum TBARS (nmol/L)</td>
<td>0.15 ± 0.03</td>
</tr>
</tbody>
</table>

Control tumor (CT), control tumor metformin (CTM), obese-MSG tumor (OT) and obese-MSG tumor metformin (OTM) (n = 10 for each experimental group).

*** p < 0.0001 vs CT.
* p < 0.05 vs CT.
** p < 0.05 vs CT.
* p < 0.05 vs OT.
Discussion

Epidemiological studies have associated obesity with a wide variety of cancers (Calle and Kaaks, 2004; Giovannucci and Michaud, 2007; Hurting et al., 2007; Jee et al., 2005) and recent data suggest that metformin can have a direct antitumoral effect or can act indirectly by improving insulin sensitivity (Evans et al., 2007; Pollak, 2007; Schneider et al., 2001). Here we show that metformin reduces the stimulatory effect of obesity on in vivo Walker-256 tumor development and increases the area of tumor necrosis.

The Walker-256 tumor we used is a breast carcinosarcoma often used in studies of anticancer therapies and tumor-induced cachexia (Fernandes et al., 1990; Folador et al., 2009); it is specific to rats and easily transplanted.

Our study is the first to show that obesity increases tumor development, the number of rats that develop tumors and neoplastic cachexia incidence. Insulin resistance, the metabolic changes and the increased generation of reactive oxygen species (ROS), assessed indirectly by the lipid peroxidation rate, may contribute to these alterations. High concentrations of diverse free radicals and oxidants generate potent reactive oxygen species (ROS) that can damage cell DNA by direct oxidation or by interfering with the mechanisms of DNA repair.
ROS may also react with proteins and lipids, forming derivative products that may alter intracellular homeostasis favoring the accumulation of mutations that, in turn, contribute to the multistage carcinogenesis process (Cejas et al., 2004; Tabuchi et al., 2006; Valko et al., 2004).

Adipose cells found in greater quantity in tumor tissues of obese-MSG rats also may have contributed to the increased tumor development in this group. Adipose cells produce and release several factors, such as leptin, IL-6 and TNF-alpha, which stimulate cell proliferation and tumor angiogenesis (Hursting et al., 2007; Somasundar et al., 2004), thus contributing to a better microenvironment for tumor development.

The finding that metformin attenuated the tumor growth only in the obese-MSG rats is intriguing. The reason is not easily explained, but it is possible that the effect of this drug on tumor cells depends on fat tissue and on the metabolic abnormalities found in obesity (Algire et al., 2008; Calle and Kaaks, 2004; Giovannucci and Michaud, 2007; Jee et al., 2005).

The observed effect of metformin decreasing the tumor development and the percentage of rats that developed tumors in obese-MSG rats may be due, at least in part, to its metabolic effects correcting lipid abnormalities, reducing accumulation of retroperitoneal and peripedidymal adipose tissues and decreasing lipid peroxidation rate.

Inhibition of lipolysis induced by metformin may be one of the mechanisms involved in the correction of the lipid profile reducing plasma triglycerides and free fatty acids (Marchetti et al., 1988). Another mechanism may involve AMPK activation (Zang et al., 2004). Activation of this enzyme induces phosphorylation and consequent inactivation of acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGC-CoA reductase), contributing to the reduction in fatty acid content and cholesterol and, consequently, the synthesis of lipoproteins.

The lower number of rats that developed tumors in the OTM group might also be explained by the reduction of the generation of ROS, assessed indirectly by the lower rate of lipid peroxidation. Metformin, owing to its antioxidant activity and to its effect decreasing the levels of circulating triglycerides, may contribute to the lower generation of ROS (Bellin et al., 2006; Cejas et al., 2004; Valko et al., 2004; Zhou et al., 2007). In fact, previous data from our laboratory have shown the lowering effect of metformin on the generation of ROS in mesenteric arteries of obese-MSG rats without tumor (Lobato et al., 2010).

Another possible mechanism to explain the effect of metformin on tumor growth is improving insulin sensitivity with consequent reduction of serum levels of this hormone. However, at least at the dose used in this study, there was no improvement in insulin sensitivity. Therefore, this mechanism can be excluded. We may raise the possibility that the effect of metformin on insulin sensitivity would be hampered by the presence of tumor cells that also decrease insulin sensitivity in peripheral tissues (Wals, 2010; Younes and Noguchi, 2000).

The fact that an increased necrotic area in tumor tissues was observed in the metformin-treated groups might also explain the reduction in tumor development in the obese rats. A direct effect of metformin on the neoplastic cell or on the vascular tissue may be leading to tumor necrosis and contributing to the decrease of tumor development. In fact, it has been proposed by Sahra et al. (2008) that metformin inhibits the proliferation of DU 145, PC-3 and LNCaP cancer cells (prostate cancer cells), decreasing cell viability, as a consequence of blocking cell cycle in G0/G1 and decreasing the levels of cyclin D1.

Therefore, metformin may have both an indirect effect correcting metabolic abnormalities and a direct effect inhibiting proliferation of neoplastic cells.

The presence of tumors in the body leads to several metabolic changes that can lead to cachexia. It is known that the more aggressive the tumor, the greater the cachexia index, which can worsen the prognosis (Wals, 2010). One possible explanation for the greater weight loss and the higher incidence of cachexia in the OT rats compared to the CT rats (OT 90% vs CT 50%) might be the high amount of energy consumed by the tumor cells, mainly in the form of glucose.

The glucose is converted to lactate by tumor cells during glycolysis, and the lactate thus produced may be used by the liver as a gluconeogenic precursor to synthesize glucose, which may be used by both the host and the tumor (Inui, 2002; Younes and Noguchi, 2000). In this process, there is greater energy expenditure because only two molecules of ATP are produced by glycolysis, whereas six molecules of ATP are utilized to synthesize glucose from lactate. This contributes to the degradation of the tissue as a result of the increased lipolysis and proteolysis as well as to weight and body mass loss leading to cachexia (Body, 1999; Tisdale, 2001; Wals, 2010; Younes and Noguchi, 2000).

The fact that metformin failed to reduce the incidence of cachexia and the greater weight loss in the OT rats can be explained by the anorectic effect of the drug described previously (Haupt et al., 1991).
and also detected in our study. Anorexia induced by metformin may prevent the maintenance of body weight in rats with tumor and favor the incidence of cachexia.

Conclusions

We demonstrated that obesity has an important role in tumor development, increasing the tumor size and volume. We also demonstrated that metformin, independent of improving insulin sensitivity, was effective in controlling tumor development. This finding led us to conclude that metformin has a direct and effective action on the tumor cell, mainly by increasing the area of tumor necrosis. But an indirect effect correcting lipid abnormalities, reducing the accumulation of periepididymal and retroperitoneal fat, and reducing lipid peroxidation rate may also be contributing to its effect.

Conflict of interest statement

The authors declare no conflict of interest.

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