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Original Paper

An Experimental Model of Diabetes and Cancer in Rats

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The aim of this study was to develop an experimental model for the study of cancer associated with diabetes. For diabetes induction, Sprague–Dawley rats were given streptozotocin (STZ, 90 mg/kg body weight (BW)), by intraperitoneal injection on the second day of life. For mammary tumour induction, rats were injected with 50 mg/kg BW of *N*-nitroso-*N*-methylurea (NMU) at 50, 80 and 110 days old. The neoplastic process and the effect of tamoxifen treatment was examined in non-diabetic and diabetic rats. The latency period, NMU-induced tumour incidence and the number of tumours per rat in diabetic rats versus controls were 117 ± 7 days versus 79 ± 9 days ($P < 0.001$); 93% versus 95% (NS); and 5.2 ± 1.6 versus 2.7 ± 0.5 ($P < 0.02$). A more benign histological pattern for tumours in diabetic animals was observed. Mammary tumours in diabetic rats grew more slowly than in controls. Tamoxifen (1 mg/kg/day) treated diabetic rats showed tumour regression in 67% of NMU-induced mammary tumours versus 53% in controls (NS). Our results show that tumour progression seems to be affected by diabetes in this experimental model. We suggest this is the result of changes to insulin-like growth factors and their receptors, which occur in diabetics, and our future research will examine this hypothesis. © 1998 Elsevier Science Ltd. All rights reserved.

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INTRODUCTION

MAMMARY TUMORIGENESIS is a complex and multisequential process during which growth factors, other oncogene products and proteins from different transduction pathways are involved [1–3]. Clinical observation of breast cancer patients with non-insulin-dependent diabetes has shown a prolonged survival and a better evolution of their oncological process than their non-diabetics peers [4–6]. The common treatment for these patients is the association of sulphonylureas for diabetes and tamoxifen (TAM) as an antitumoral agent [4].

Insulin, insulin-like growth factors (IGFs) and their corresponding receptors (IGF-IR and IGF-IIR) participate in the growth and proliferation of normal and neoplastic mammary cells [7–9]. Plasma IGFs and their corresponding binding proteins are modified in diabetic patients [10–12], but these variations have not been studied when both pathologies

(diabetes and cancer) coexist. In contrast, TAM down-regulates IGF expression in plasma and tissues [13,14] influences plasma levels of IGF-I and IGF binding protein-1 in breast cancer patients [15]. Sulphonylurea derivatives have also been shown to modify some qualitative and quantitative characteristics of IGFs and their receptor proteins [16].

Pathological changes in cell growth and differentiation present in diabetic tissue are dynamically governed, at least in part, by IGFs and studies in diabetic patients have demonstrated abnormalities in IGF regulation [17]. The appearance of neoplasia in diabetic patients and the behaviour of IGFs in such conditions has not been sufficiently investigated and prompted us to develop an experimental model in rats to explore some biological characteristics of mammary neoplasias in diabetic animals. Results obtained by Cohen and Hilf [18] demonstrated that a proportion of 7,12-dimethylbenzo-(a)anthracene (DMBA)-induced mammary carcinomas in rats regressed after the tumour bearing animals have been made diabetic [19].

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Accordingly, the objective of this study was to obtain an experimental model for the study of interrelations between different biological parameters in cancer associated with diabetic disease. Available experimental models of induced diabetes were reviewed [20–24] choosing the induction of non-insulin-dependent diabetes by an injection of streptozotocin (STZ) into rats on the second day of life [21]. Mammary tumours were induced by intraperitoneal (i.p.) injections of *N*-nitroso-*N*-methylurea (NMU) as previously reported [25–27]. Parameters of tumour development and of the diabetic process were characterised, and the effect of TAM treatment on normal and diabetic rats was examined.

MATERIALS AND METHODS

Reagents

NMU was synthesised in our laboratory as previously described [25]. Tamoxifen citrate was kindly provided by Gador S.A. (Buenos Aires, Argentina). STZ was purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A) and reagents for insulin radioimmunoassay (RIA) from International CIS (France). A blood glucose micromethod (Glucometer Gx, Ames S.A., Argentina) was used for determination of serum glucose levels. Glycosylated haemoglobin was determined by a chromatographic spectrophotometric test (Biosystems, Argentina).

Animals

Virgin female Sprague–Dawley rats inbred in our laboratory were randomly separated into batches and housed in stainless steel cages, with water and food *ad libitum*, at a temperature of 22–23°C, humidity of around 56% and a 12 h light cycle. Their oestrous cycle was monitored by microscopic observation of vaginal smears from 45 days of life.

Diabetes induction

On the second day of life (28–36 h after birth), the rats were i.p. injected with 90 mg/kg STZ in 0.09 M citrate buffer pH 4.8, according to the method of Weir and associates [21].

At day 45, all rats were subjected to a glucose tolerance test: after basal blood glucose determination, 2 g/kg body weight (BW) of glucose was i.p. injected and blood glucose levels determined at 30, 60 and 120 min. Animals showing abnormal glucose tolerance curves were selected for diabetic groups.

Tumour induction

Rats, both control and diabetic groups, were i.p. injected with three doses of NMU at 50, 80 and 110 days of life as previously described [25]. For mammary tumour detection and growth control, animals were examined by palpation three days a week during the experimental period; body weight was also observed.

In vivo studies

Thirty-two rats were equally divided into two groups: A and B. Group A rats (normal) were injected with three i.p.-NMU doses [25]. Animals from group B (diabetic) were injected on the second day of life with subcutaneous (s.c.) STZ [21]; rats with abnormal glucose tolerance curves were injected with NMU in similar conditions to group A. The parameters recorded were: (a) latency period, the number of days between the first NMU injection and the appearance of the first tumour; (b) tumour incidence, the percentage of

animals that developed at least one tumour; (c) tumour number per rat, the number of tumours per rat in animals developing at least one tumour. The oestrous cycle was monitored by means of microscopic observation of vaginal smears. At death, the mammary tumours, pancreas and other tissues were removed for histopathological studies. Serum samples were frozen for insulin measurement by RIA.

Additionally, for studying evolution of diabetes in STZ-injected rats, histological observation of the pancreas was performed in a further 30 rats (15 controls and 15 STZ-injected). Serum glucose, glycosylated haemoglobin and insulin levels were determined and the oestrous cycle observed for correlation with histological findings when rats were killed at 3, 5 and 6 weeks of life in groups of five.

To study tumour behaviour after TAM treatment in both normal and diabetic rats bearing induced mammary tumours, other experiments were performed. Table 1 shows treatment schedules for groups 1 to 4. TAM, 1 mg/kg/day, was suspended in corn oil and s.c. injected. Parameters recorded were: tumour growth rate and the percentage of regressing tumours; the observation period was 60 days. Each tumour was classified as regressing, growing or stable according to its size in relation to pretreatment. More than one category of response in each animal could be observed when more than one tumour was present.

Plasma insulin levels

Serum samples from rats of groups A and B and from those killed at 3, 5 and 6 weeks of life, were frozen at –70°C. Insulin levels were measured by RIA, performed in duplicate.

Glycosylated haemoglobin

Glycosylated haemoglobin was determined as the percentage of haemoglobin-A_{1C} (HbA_{1C}) of total haemoglobin.

Histopathological studies

At the completion of the experiments, animals were subjected to necropsy. Samples of tumour and tissues, such as the pancreas, uterus, ovaries, lung, liver, spleen, kidney and intestine, were harvested for histological examination. Specimens were fixed in 10% formaldehyde and embedded in paraffin. Haematoxylin–eosin (HE) and Alcian blue–Zafranin stained sections were microscopically examined.

Table 1. Tamoxifen treatment of normal and diabetic rats with mammary *N*-nitroso-*N*-methylurea (NMU)-induced tumours. Groups 1 and 2, normal rats with mammary intraperitoneal (i.p.) NMU-induced tumours. Groups 3 and 4, diabetic rats with i.p. NMU-induced tumours. Groups 2 and 4, rats treated with tamoxifen (TAM). Treatment began when at least one of the induced tumours reached a diameter of 1.5 cm and continued for 60 days

Group	Drugs and route of administration	Doses mg/kg
1	NMU, i.p.	50
2	NMU, i.p. + TAM, s.c.	50 + 1/day
3	STZ, s.c. + NMU, i.p.	90 + 50
4	STZ, s.c. + NMU, i.p. + TAM, s.c.	90 + 50 + 1/day

i.p., intraperitoneal; s.c. subcutaneous; NMU, *N*-nitroso-*N*-methylurea; STZ, streptozotocin; TAM, tamoxifen.

Statistical analysis

The latency period was analysed by one-way variance analysis and the Newman–Keuls test [28]. Statistical significance of the tumour number per rat was analysed using the non-parametric Kruskal–Wallis test [29]. Tumour incidence and tumoral regression were compared by means of the χ^2 test [28]. RAI data are expressed as means \pm SEM and statistical significance calculated by one-way variance analysis and the Newman–Keuls test [28].

RESULTS

Diabetes induction

Serum glucose levels were determined in normal and diabetic rats. In normal rats at 45 days of life, the mean value was 80 ± 11 mg/dl (group A) and in STZ-injected rats (group B) it was 118 ± 28 mg/dl (NS). Eighty-eight per cent (14/16) of STZ-injected rats showed an abnormal glucose tolerance curve (Figure 1), with glucose levels persistently higher than in normal rats up to 120 min post-glucose injection. In agreement, histological observation of the pancreas from rats of group B showed scarce and small Langerhans' islets, scarce endocrine cells with reduced cytoplasm, capillaries and stromal cells (Figure 2a). In contrast, the control rats showed acini and Langerhans' islets with normal structure and size, with rounded cells and abundant cytoplasm, arranged in cords detached from capillaries (Figure 2b).

No significant difference between normal and diabetic rats was observed in glycosylated haemoglobin values: $6.9 \pm 2.1\%$ versus $7.4 \pm 0.4\%$, respectively.

Tumour induction

Tumour growth parameters at 140 days of life in diabetic and non-diabetic rats are listed in Table 2. Tumour incidence was not significantly different in control and STZ-injected batches. The latency period and the tumour number per rat were markedly different between the groups (A versus B), diabetic rats showing a longer latency period ($P < 0.001$) and a lower tumour number per rat ($P < 0.02$).

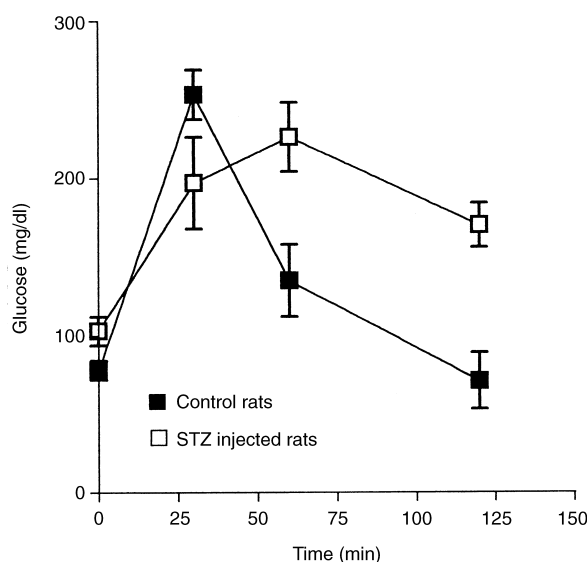


Figure 1. Glucose tolerance test. Serum glucose levels (mg/dl) of streptozotocin (STZ)-injected (\square) and control (\blacksquare) at 30, 60 and 120 min after intraperitoneal body weight 2 g/kg injection of glucose. Error bars show 95% confidence intervals.

Table 2. Latency period, tumour incidence and tumour number per rat in control (A) versus diabetic (B) rats, at 140 days before the first intraperitoneal-N-nitroso-N-methylurea (NMU) injection

	Latency period (days)	Tumours/rat	Tumour incidence (%)
A (NMU)	79 ± 9	5.2 ± 1.6	95
B (STZ + NMU)	$117 \pm 7^*$	$2.7 \pm 0.5^\dagger$	93^\ddagger

NMU, N-nitroso-N-methylurea; STZ, streptozotocin.

* $P < 0.001$, one-way variance analysis and Newman–Keuls test.

$^\dagger P < 0.02$ versus NMU, χ^2 test. $^\ddagger P$: NS versus NMU, Kruskal–Wallis test, for B versus A, respectively.

Histopathology of induced tumours

Histological observation showed evident differences in the growth pattern of tumours from groups A and B. All tumours from group A were malignant carcinomas with cribriform, comedo and/or papillary patterns, pleomorphism and numerous atypical mitoses, anisocarioses and solid tumoral areas with scarce secretion (Figure 3a). In contrast, group B tumours showed a more benign pattern in most cases, with a tendency to differentiation and ductal secretory structures with a single layer of flat cells. Mitosis and diffuse stromal infiltration were present to a lesser degree and solid tumour areas were not observed (Figure 3b). Yellow bodies in the ovaries (Figure 4a) and proliferate epithelium in the uterus (Figure 4b) of group B rats were present in agreement with microscopic observation of vaginal smears, indicating a

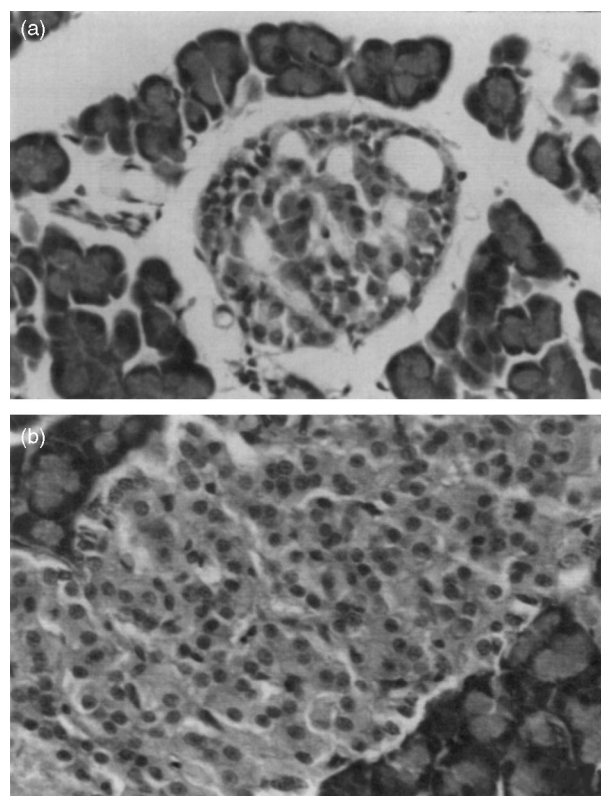


Figure 2. (a) Pancreatic Langerhans' islets with loss of different cell type components showing remnants of ducts and main cells, scarcity of β -cells and dilated ducts (magnification: $400\times$, HE). (b) Normal pancreatic Langerhans' islets (magnification: $400\times$, HE).

normal reproductive cycle. Lung, liver, spleen, kidney and intestine failed to show injury or atypical signs.

Young normal and STZ-injected rats were killed at weeks 3, 5 and 6 of life for studies of diabetic evolution. The pancreas pattern in normal or STZ-injected animals showed slight differences at the third week, which were more pronounced at the sixth week, with a lower number and decreased size of Langerhans' islets than in normal rats.

Plasma insulin levels

No significant differences in plasma insulin levels between control and diabetic groups were seen ($10.9 \pm 4.5 \mu\text{UI/ml}$ versus $8.3 \pm 3.0 \mu\text{UI/ml}$, respectively). Young rats (3, 5 and 6 weeks of life) failed to show significant differences.

Tumoral regression studies

TAM treatment of normal and diabetic rats bearing i.p. NMU-induced mammary tumours failed to show significant tumoral growth differences (Table 3). None of the tumours in the control group (group 1) showed regression and only one of the 49 was stable. In the diabetic rats (group 3) no evidence of spontaneous tumour regression was seen, but 4/23 (17%) of these tumours were stable. Fifty-three per cent (24/45) of TAM-treated tumours (group 2) showed regression as in previous experiments [27, 30] and when diabetic rats were treated with TAM (group 4), 12/18 (67%) of tumours regressed and 2/18 (11%) were stable.

Histopathological studies of tumours in control and diabetic rats (group 1) showed no intratumoral necrosis. Tumours regressing under TAM treatment in normal and

Table 3. Tumoral evolution in normal and diabetic rats with intraperitoneal N-nitroso-N-methylurea (NMU)-induced tumours under tamoxifen (TAM) treatment for 60 days

Group	Regressing n (%)	Stable n (%)	Growing n (%)
1 NMU (n = 49)	0 (0)	1 (2)	48 (98)
2 NMU + TAM (n = 45)	24 (53)*	1 (2)	20 (44)
3 STZ + NMU (n = 23)	0 (0)	4 (17)	19 (83)
4 STZ + NMU + TAM (n = 18)	12 (67)*†	2 (11)	4 (22)

NMU, N-nitroso-N-methylurea; STZ, streptozotocin; TAM, tamoxifen. * $P < 0.001$ versus NMU, χ^2 test. † P : NS versus NMU + TAM, χ^2 test.

diabetic rats showed 80–90% necrosis and evidence of glandular hypersecretion (groups 2 and 4).

Figure 5 illustrates that the tumours in normal rats which continued to grow reached a size three to four times greater than the pre-treatment size at the end the observation period (60 days) (Figure 5, control). Tumours in diabetic rats grew at a much slower rate, reaching a final size at the most twice the initial pretreatment size (Figure 5, STZ). In rats given TAM treatment, no significant difference was observed in tumour growth rate for tumours that continued growing in either normal or diabetic rats (TAM(g) and STZ + TAM(g), respectively) with respect to controls. Tumours that regressed under TAM treatment showed a similar growth rate in

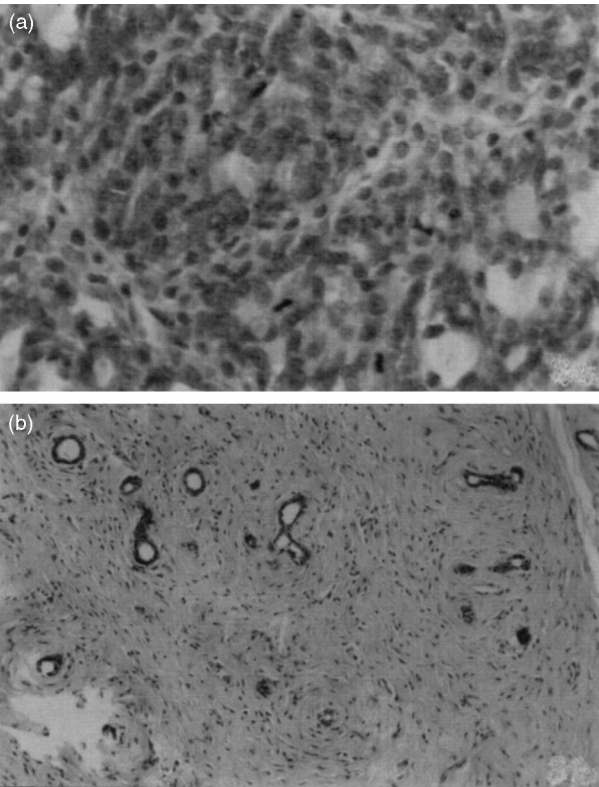


Figure 3. (a) Cribiform adenocarcinoma with striking cell growth from a non-diabetic rat. Frequent mitosis and marked anisocariosis (magnification: 400 \times , HE). (b) Benign fibroadenoma from a diabetic rat (magnification: 100 \times , HE).

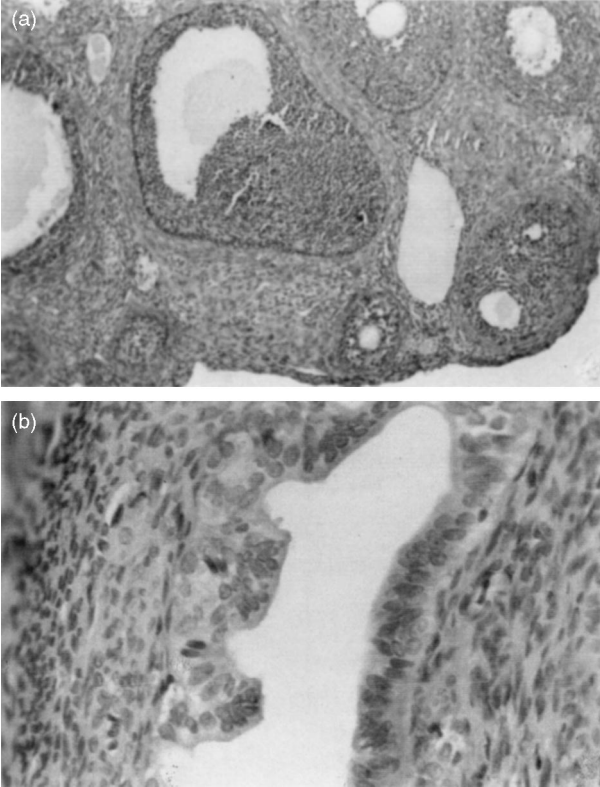


Figure 4. (a) Ovary with numerous follicular structures in diverse maturation stages from a diabetic rat (magnification: 100 \times , HE). (b) Typical endometrial gland with markedly proliferative epithelium from a diabetic rat (magnification: 400 \times , HE).

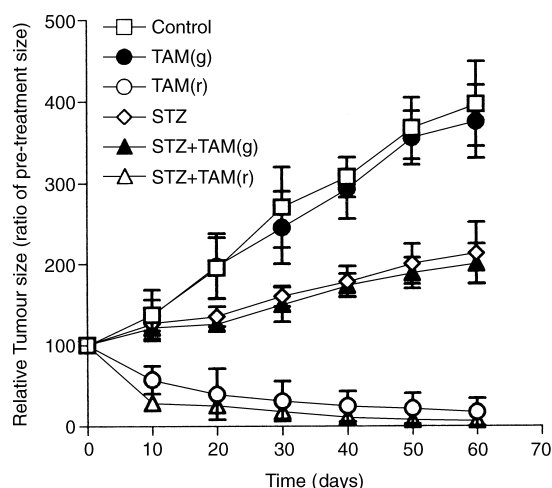


Figure 5. Tumour growth rate in streptozotocin (STZ)-injected and normal rats under tamoxifen (TAM) treatment. Observation period: 60 days. Control, tumours of non-treated rats. STZ, tumours of diabetic rats. TAM(g), tumours growing on TAM-treated rats. TAM(r), tumours regressing in TAM-treated rats. STZ(g), tumours growing on TAM-treated diabetic rats. STZ(r), tumours regressing on TAM-treated diabetic rats.

normal and diabetic animals (TAM(r) and (STZ + TAM(r), respectively).

Finally, all normal and diabetic rats showed a regular oestrus cycle. In contrast, TAM-treated animals from normal and diabetic groups showed an irregular reproductive cycle, mostly arrested in dioestrus.

No significant differences in body weight for normal and diabetic rats were found.

DISCUSSION

Experimental and clinical research indicates a crucial role of insulin in the development of mammary tumours [18]. In our paper, the latency period was longer and the number of tumours per rat was lower in diabetic rats than in controls, indicating a less aggressive neoplastic process in diabetic rats. Histopathological observations also indicated fewer atypical cells and mitoses in tumour samples from diabetic rats, as well as lower cellular indifferenciation. This contrasting pattern with respect to NMU-induced tumours under normal conditions was independent of gonadal function, which was normal in the diabetic group. Tesone and colleagues [31] described ovarian dysfunction and several biochemical alterations in STZ-induced diabetes in adult rats, with lower circulating levels of progesterone, decreases in luteinising hormone (LH) receptors and other alterations in male reproductive functions [32]. In our experiments, circulating follicular stimulating hormone (FSH) and LH values were not determined, but there was no evidence of altered ovulation appearance or proliferation of the uterus epithelium (Figure 4); the cytotoxic effect of STZ on pancreatic β -cells seems to lack any detectable repercussion on reproductive function. The hormonal milieu, crucial for the development of these mammary tumours [27], appeared to be normal throughout the carcinogenesis process. In our experiments, the results cannot be attributable to insulin levels because they were normal for the entire observation period. Our explanation for the obtained results is that some changes in IGFs, their corresponding receptors and/or binding proteins

could affect tumour promotion/progression phases of carcinogenesis, after the initiation step triggered by NMU. As a result, tumours develop with a greater latency period, a lower tumour number per rat, lower tumour growth rate and more benign pattern than in controls. The histologically benign pattern in these STZ diabetic animals resembles that previously observed in NMU-induced mammary tumours in animals pretreated with TAM [30], suggesting inhibition or interruption of the promotion/progression process and could be a result of diabetes.

In the present work, results of normal and diabetic rats treated with TAM failed to show significant differences, although a positive trend was observed for the diabetic group (53% versus 67% of tumours showed regression). It is known that TAM is the most common anti-oestrogen employed in patients with hormone-dependent breast cancer [33] acting as an oestrogen antagonist and also by other mechanisms [34, 35]. We reported previously that all i.p. NMU-induced mammary tumours expressed oestrogen and progesterone receptors, but only 50–53% regressed under TAM treatment [27, 30]. Thus, the observed trend to higher tumour regression in diabetic rats could be attributable to specific effects of TAM on the IGF system or to a more benign tumoral pattern observed in diabetic animals. Rhomberg demonstrated that metastasising breast cancer in women who also had diabetes took a protracted course and survival times were significantly longer in women with diabetes [6]. More recently, several studies have shown that TAM treatment causes a moderate decrease in total plasma IGF-I [13, 36, 37], but its action on IGF-binding protein is controversial [38]. De Cupis and associates [39] provided evidence that, in oestrogen receptor-positive human breast carcinoma cell lines, steroidal anti-oestrogens inhibit cell growth and modulate the IGF-I mitogenic system by means of a mechanism not yet identified. Many studies indicate that IGF-IR plays a central role in the mechanism of neoplastic transformation [9]. IGFs and their corresponding receptors and binding proteins have also been shown to be affected in diabetic organisms and tissues [40, 41]. Interrelations between oestrogen, IGFs and their receptors play a crucial role in normal and neoplastic cell growth which have been found to be altered in diabetes. Sulphonylureas, used as hypoglycaemic drugs, have been reported to modify IGF-IGF-R binding and their association with TAM treatment could result in a significant enhancement of antitumoral action as reported in clinical studies [3]. Our subsequent experiments will investigate the effects of the combination treatment of sulphonylureas plus TAM.

The experimental model described in this paper appears to be appropriate for studying the role of IGFs and their corresponding receptors in relation to the hormonal status of mammary neoplasia. Our future research will aim to examine tumour pathogenesis and treatment in diabetic subjects as well as the interrelation between anti-oestrogen therapy and IGFs.

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