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On the Origin of Induced Pancreatic Islet Tumors: A Radioautographic Study (42471)

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Abstract. Endocrine tumors of the pancreas are induced in a high percentage of young rats by injections of streptozotocin and nicotinamide (SZ/NA). Benign tumors first appear 20 to 36 weeks after drug injections. To determine the possible site of their origin, the incorporation of [^{3}H]thymidine into islets, ducts, acini, microtumors, and gross tumors was examined by radioautography of histologic sections at 1 to 36 weeks after drug injection. Drug treatment led to early (1- to 6-week) increases in nuclear ^{3}H labeling of exocrine pancreatic structures (ductal and acinar cells), which may involve DNA repair processes. A secondary increase in labeling of duct cells during the period of tumor emergence supports the assumption that SZ/NA-induced tumors are of ductal origin. Microtumors and gross tumors also exhibited markedly elevated rates of [^{3}H]thymidine incorporation compared to control islets. Nontumorous islet tissue, which exhibited a gradual decrease in volume due to B-cell destruction by the drug injection, showed about 10-fold higher ^{3}H labeling than islets of controls at all time points. The results suggest that in addition to ductal precursors, islets that survive SZ/NA-induced injury may also provide sites of focal endocrine cell differentiation to tumor tissue. Once established, both microtumors and gross tumors continue to grow by accelerated cell division.

Spontaneously occurring pancreatic islet adenomas are observed rarely in cattle (1), dogs (2), rats (3), Syrian hamsters (4), and mice (5). When rats are exposed to neutrons (3) or given pyrrolizidine alkaloids (6, 7) the incidence of islet cell tumor formation is increased to 20% after 22 months. Rakieten *et al.* (8) first reported a 60% incidence of islet adenomas in rats 8 to 9 months after the administration of streptozotocin (a diabetogenic agent) and nicotinamide.

Most spontaneous and drug induced islet tumors in adult animals are believed to arise from ductal precursor cells (i.e., the same or similar cells that give rise to islet cells during prenatal development), but other sources are possible. For instance, the presence of abundant mitotic figures in the islet tumors of golden hamsters indicates that these cells may also develop by the proliferation of mature endocrine cells (9). In contrast, mitotic figures in human tumors are reportedly rare (10, 11). Because of the high rate of induction afforded by streptozotocin/nicotinamide (SZ/NA) treatment, and the growing body of background information on the development, morphology, and physiology of this tumor model, we addressed the question of tumor origin by examining the incorporation of [^{3}H]thymidine into endocrine, ductal, and acinar tissues of rats at various intervals after drug administration. We expected that cells showing elevated incorporation of [^{3}H]thymidine would be undergoing proliferation and thus be implicated in endocrine cell neogenesis.

Materials and Methods. *Tumor induction.* The method for inducing pancreatic islet tumors is as described earlier (12–14). Twenty-nine 6-week-old male Holtzman rats (160–180 g body wt) were fasted overnight and injected ip with nicotinamide (NA; 350 mg/kg body in saline). Ten minutes later, a single iv injection of streptozotocin (SZ; 65 mg/kg in citrate buffer, pH 4.5) was given followed by another ip injection of NA 180 min after the first dose. In addition, 20 rats were injected with NA and citrate buffer, and served as controls. The experimental and control animals were killed at 1, 6, 20, and 36 weeks after the injections.

Injection of [^{3}H]thymidine. Twenty-four hours before the animals were killed, [6-^{3}H]thymidine (sp act 23 Ci/mmole; New England Nuclear, Boston, MA) was injected by intraperitoneal route (1 μCi/g body wt). After sacrificing the animals by guillotine, the pancreas and a portion of jejunum were ex-

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cised and fixed in Bouin's fluid for 12–16 hr, and the tissues were processed through alcohol and solvent stages in an Autotechnicon and finally embedded in paraffin. Each paraffin block was serially sectioned at 4 μm. Every hundredth section was affixed to gelatinized slides to ensure adhesion of the photographic emulsion and to reduce stress artifacts due to lateral displacement of the emulsion during drying (15). The slides were exposed to 2% perchloric acid for 5 min during the hydration procedure to remove RNA which may have incorporated a small amount of label (16).

In a dark room the slides were dipped into NTB-3 nuclear track emulsion (Eastman Kodak, Rochester, NY), air dried, and then kept in a light-proof box with desiccant at 4°C for 21 days before developing (Kodak D-19 Developer). The sections were counterstained with Delafield's haematoxylin, dehydrated, and coverslipped.

One slide containing a section of jejunum from each rat was included to evaluate the status of injection and uptake of the labeled thymidine. In addition one slide not containing the tissue was coated with emulsion to test both the emulsion and the developer.

Quantification of labeled cells. The average background level was determined at 10 random locations in each slide, over an area without tissue and approximately equal to the size of the cell nucleus. If the nucleus of a cell contained more than five grains above background level it was scored as labeled.

For each animal a minimum of 1000 endocrine or duct cells were examined and scored as labeled or unlabeled. A minimum of 1000 acinar cells within a depth of five cell layers from the islets were examined. This procedure ensured reliability of counting the labeled acinar cells since it is reported that the rate of acinar cell labeling is elevated in the immediate vicinity of islet tissue (17).

The thymidine uptake rate was calculated as

$$\% \text{ labeled cells} = \frac{\text{number of labeled cells}}{\text{number of cells counted}} \times 100.$$

Control and experimental values for cell labeling were compared by the Neuman–Keuls test (18).

Results. The body weights and pancreatic weights of SZ/NA-treated and control rats are given in Table I. No significant differences were observed between treated rats and their age-matched controls. This suggests that the overall growth of the treated rats was not noticeably affected by drug injection, so that [^{3}H]thymidine labeling in both groups could be compared directly.

Uptake of tritiated thymidine in acinar cells. In control rats the rate of tritiated thymidine uptake in the acinar cells was 0.84% ± 0.18 in all animals sacrificed at different time periods, and hence values for control rats were pooled (Fig. 1). In contast, after SZ/NA injection thymidine uptake by acinar cells was significantly higher at 1 and 6 weeks; values returned to control at 20 and 36 weeks (Fig. 1).

Duct cells. In control rats the thymidine uptake (0.82% ± 0.18) in duct cells was not significantly different at various time periods after injection of buffer, so values for these animals were pooled (Fig. 2). By contrast there was a significantly higher uptake of thymidine label by the duct cells of SZ/NA-treated rats after 1, 6, and 36 weeks. At 20 weeks, although thymidine uptake was slightly elevated (1.42 ± 0.32%), it was not significantly higher than controls.

TABLE I. BODY AND PANCREATIC WEIGHT OF CONTROL AND SZ/NA-TREATED RATS AT VARIOUS TIME PERIODS

	Control			SZ/NA Treated		
Weeks post injection	No. animals	Body weight (g)	Pancreatic weight (g ± SEM)	No. animals	Body weight (g)	Pancreatic weight (g ± SEM)
1	5	275	0.86 ± 0.02	5	266	0.87 ± 0.03
6	4	392	1.15 ± 0.04	6	359	1.09 ± 0.03
20	5	494	1.43 ± 0.04	6	471	1.40 ± 0.03
36	6	546	1.53 ± 0.04	12	558	1.52 ± 0.03

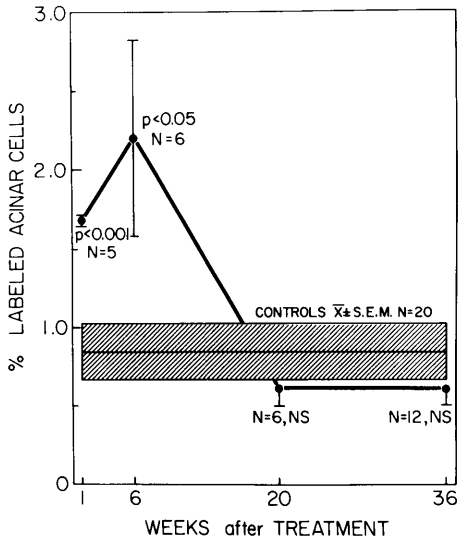

FIG. 1. The uptake rate of [³H]thymidine by acinar cells from streptozotocin/nicotinamide (SZ/NA)-treated and control rats as determined by radioautography.

The ratio of thymidine uptake by duct cells to acinar cells is given in Table II. In control rats at all time periods of observation this ratio was 1.02 ± 0.05. In SZ/NA-treated rats, this ratio was 1.17 ± 0.08 and 1.08 ± 0.23 at 1 and 6 weeks post injection, and 1.96 ± 0.42 and 4.10 ± 0.92 at 20 and 36 weeks post injection. These data indicate that proliferation of duct epithelium far exceeded that of acini in the later periods of observation.

Endocrine cells. The uptake rate of labeled thymidine in all endocrine tissue is shown in Fig. 3. In control rats the uptake rate varied with age of the animals. At 1 week after commencement of the experiment (7-week-old control rats) the uptake rate was 1.16 ± 0.11%, which steadily declined with age and at 36 weeks it was 0.07 ± 0.02%. However, in SZ/NA-treated rats the uptake rate was significantly higher than that for controls at all time periods. The sudden rise in the uptake from 20 to 36 weeks coincided with the appearance of grossly visible and microscopic tumors in the randomly selected slides of pancreatic section (14). The actual rate of thymidine labeling in treated rats was underestimated, since data from rats that did not contain gross or microscopic tumors were included in computation. Of particular importance is the observation of significantly elevated [³H]thymidine incorporation into endocrine tissues of SZ/NA-treated rats throughout the period of observation.

Tritiated thymidine uptake by islets, microtumors, and gross tumors of SZ/NA-treated rats. Of the 12 animals sacrificed 36 weeks after injection of the combined drugs, seven animals contained microscopic and grossly visible tumors (over 2 to 3 mm in diameter) and five had microscopic tumors only. Microscopic tumors are "oversized" islets (with diameters ranging between 0.5 and 1.0 mm) which were clearly discerned in histologic sections of SZ/NA-treated rats (14) and also after islet isolation (19). Besides larger size, microtumors have a random arrangement of endocrine cells. All SZ/NA-treated rats also contained numerous islets of Langerhans, which contained a core of B cells with a mantle of A, D, and PP cells.

As shown in Fig. 4, islets of control rats exhibited uptake of label at a rate of only 0.07 ± 0.02% at 36 weeks, whereas islets of the SZ/NA-treated rats showed a significantly higher uptake of 1.5 ± 0.40%. Microscopic tumors had an uptake of 6.4 ± 1.3%, whereas the gross islet adenomas had an uptake rate of 9.7 ± 1.0%. Thus, the drug treatment carried out 36 weeks earlier significantly enhanced the

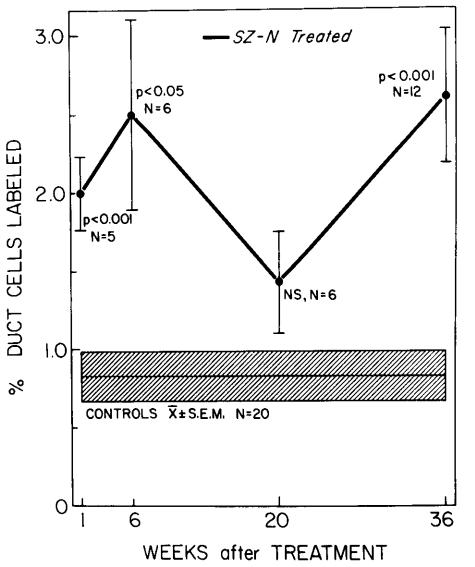

FIG. 2. The uptake rate of [³H]thymidine by duct cells from SZ/NA-treated and control rats as determined by radioautography.

TABLE II. RATIO OF [^{3}H]THYMIDINE LABELING OF DUCT TO ACINAR CELLS IN CONTROL AND SZ/NA-TREATED RATS

	Weeks post drug treatment							
	1		6		20		36	
Treatment group	No. animals	Ratio SEM	No. animals	Ratio SEM	No. animals	Ratio SEM	No. animals	Ratio SEM
SZ/NA	5	1.17 ± 0.08	6	1.08 ± 0.23	6	1.96 ± 0.42	12	4.10 ± 0.92
Control	20	1.02 ± 0.05						
P		NS		NS		<0.05		<0.005

uptake of thymidine in all endocrine tissue. The relatively higher rates of thymidine uptake by microtumors and grossly visible tumors suggest continued growth of these organs after their initial appearance.

Discussion. Incorporation of labeled thymidine into the nucleus during the S phase of the cell cycle is widely used as an indicator of mitosis (16). It has been suggested also that some thymidine incorporation is indicative of DNA turnover (20) and/or repair (21, 22). Significant incorporation of thymidine for DNA turnover, however, is generally refuted (16).

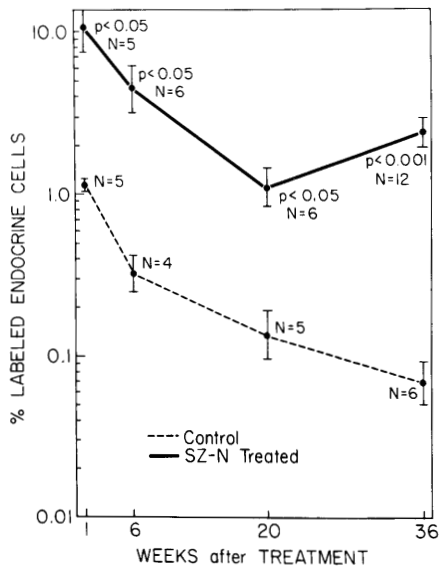

FIG. 3. The uptake rate of [^{3}H]thymidine by endocrine cells of SZ/NA-treated and control rats as determined by radioautography. Endocrine cells from both islets of Langerhans and tumor masses were included in computation. Note the log scale in ordinate.

Extensive DNA repair and synthesis are believed to occur in all mammalian cells, including embryonic and tumor lines, following the alkylation of the DNA bases (21, 23). If alkylation of DNA is not reversed by rapid DNA repair and synthesis, the damage may culminate in mutations and eventually in carcinogenesis. Streptozotocin (SZ) and methyl nitrosourea are recognized mutagenic and carcinogenic agents, possibly because of their ability to alkylate DNA. Not surprisingly, SZ alone can produce islet adenomas in surviving diabetic animals (24). Perhaps in conjunction with nicotinamide the drug has an even more observable effect on DNA repair, since many B cells survive the initial cytotoxic insult.

It has been reported that chromatin breaks induced by nitroso compounds begin repair within 48 hr, and the process is completed by 9 days (25, 26). Specifically, administration of SZ alone to mice and guinea pigs enhanced the mitotic rate of islet B cells within the first 2 weeks (27). In rats some regeneration of B cells has been observed from 4 to 10 months following SZ treatment (28). In 5- to 6-week-old rats, injection of SZ enhanced tritiated thymidine incorporation in the islet cells at 4 days but not at 7 days (29); no increase in the labeling of acinar cells was observed at any time (29). The transitory increase in mitotic activity was attributed to compensatory hyperplasia of surviving B cells following SZ inflicted injury. In view of these observations the significantly higher number of labeled acinar, duct, and endocrine cells 1 week after SZ/NA injection could be attributed to DNA repair. However, it is difficult to envision the enhanced uptake of label by ducts and islets at 6 weeks following drug treatment as due only to repair processes. The continued high

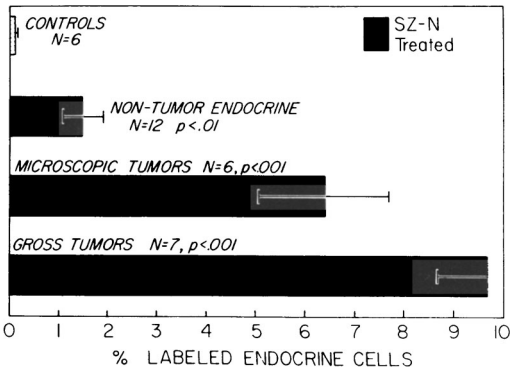

FIG. 4. The uptake rate of [^{3}H]thymidine by endocrine cells of specific organs of SZ/NA-treated and age-matched control rats 36 weeks after drug treatment, as determined by radioautography.

rate of labeling in islets of Langerhans at 6 and 20 weeks after SZ/NA treatment, albeit at a lower level than at 1 week, probably represents accelerated cell division. Theoretically, this should lead to an increase in total islet mass. A recent morphometric study clearly demonstrated, however, that the pancreatic islet volume was significantly decreased compared to controls at these time periods (14). Specifically, the aggregate (total) B-cell volume of drug-treated rats was decreased from controls by 20% at 6 weeks, by 51% at 20 weeks, and by 53% at 36 weeks post injection. These conflicting findings may be explained by assuming that the cell attrition rate in surviving islets is overwhelmingly greater than the rate of cell genesis. By 36 weeks after the drug treatment the total endocrine mass was found to be greatly increased due to islet tumor formation (14). This coincides with the very high rate of duct and endocrine cell labeling (Figs. 2, 3). Because the labeling rate of acinar cells was constant at 20 and 36 weeks they apparently did not contribute to the increase in total endocrine mass at 36 weeks.

Thymidine incorporation in duct and acinar cells is considered a measure of the generative potential of these cells. In control animals the rates of labeling in acinar and duct cells were found to be 0.84 and 0.82%, respectively (Figs. 1, 2). These figures agree closely with the values reported by other workers (30, 31). The pancreatic size steadily increased with age both in the control and SZ/NA-treated rats, as shown in Table I. This increase in tissue size can account partially for the presence of thymidine label in acinar and duct cells of the control rats at various time periods. Since the acinar and duct cells form a functional unit for the synthesis and secretion of digestive enzymes, it is likely that these two cell populations would increase at approximately the same rate and the ratio of duct to acinar cell labeling would be constant. Indeed in the control animals at all ages studied, the ratio of thymidine uptake rates of duct to acinar cells showed a value of 1.02 compared with 1.17 and 1.08 in the SZ/NA-treated animals at 1 and 6 weeks post treatment. But at 20 and 36 weeks the respective ratios were significantly higher in treated rats, namely 1.96 and 4.10 (Table II). On this basis it is possible to suggest that ducts may be one source of protodifferentiated cells which may develop into new endocrine cells. On the other hand, this increased rate of [^{3}H]thymidine incorporation may not involve islet progenitor cells, but only reflect the induction of duct cell growth by SZ/NA injections. Although not quantitated, exaggerated proliferation of ductular profiles was observed histologically in drug-treated rats.

The rate of labeling of the endocrine cells of control rats was 1.16% at 1 week following the commencement of the experiment and 0.33% at 6 weeks. Messier and LeBlond (30) and Schultze and Oehlert (31) reported mitotic indices of 0.5% to 1.0 and 0.8%, respectively, in rat islet tissue. Consistent with other studies (32, 33), we found that the labeling rate in the control rats rapidly decreased to 0.07% at 36 weeks (42-week-old rat), indicating a very low mitotic rate in islets of older rats. In SZ/NA-treated rats the rate of labeling in tumor endocrine cells at 36 weeks was markedly greater, signifying that the increase in the tumor volume at that point in time was largely due to cell regeneration.

Source of the induced islet adenoma. In embryonic and neonatal life and in adults, endocrine cells appear to arise from precursor cells in pancreatic ducts (32–35). In insulinomas, endocrine cells have been reported to bud off the pancreatic ducts, and the close proximity of ducts with tumors is considered sufficient evidence for their ductal origin (10, 38). Data presented here also seem to indicate that ducts may be involved in endocrine cell

proliferation. At 36 weeks after drug treatment, the number of labeled cells in ducts was over threefold greater than in controls. In addition, collections of ducts within or surrounding the tumor tissue and, rarely, of immunohistochemically stained insulin-producing cells in ducts suggests their ductal origin (33, 38, 39).

Based on the high rate of [^{3}H]thymidine labeling in tumors, microtumors, and islets of SZ/NA-injected animals (9.7, 6.4, and 1.5%, respectively) compared to control rat islets (0.07%), it is reasonable to consider that the development of islet tumors occurs also in part by mitosis in islet endocrine cells. This conclusion is supported by unpublished observations of mitotic cells in SZ/NA-treated islet tissue and aldehyde fuchsin-stained B cells undergoing mitosis.

Thus, the present radioautographic study, rather than confirming the exclusive ductal origin of SZ/NA-induced islet adenomas, raises the possibility of an islet origin for these tumors. Although the total pancreatic volume of islets of Langerhans progressively decreased (14), the active turnover of islet cells (as revealed by [^{3}H]thymidine incorporation rates) may provide the opportunity for focal tumor formation. Further studies are necessary to establish the specific pancreatic site or sites of primary endocrine cell neoplasia, and the factors responsible for its initiation.

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