International Journal of Pancreatology, 4 (1989) 127–137 $02.20

Histogenesis of Early Preneoplastic Lesions Induced by *N*-Nitrosobis(2-oxopropyl)amine in Exocrine Pancreas of Hamsters

*M. Meijers, J. P. Bruijntjes, E. G. J. Hendriksen, and R. A. Woutersen**

TNO-CIVO Toxicology and Nutrition Institute, Department of Biological Toxicity, PO Box 360, 3700 AJ Zeist, Netherlands

Received April 14, 1988; Revised June 8, 1988; Accepted June 13, 1988

Summary

The histogenesis of early putative preneoplastic lesions, arising in exocrine pancreas of Syrian hamsters after treatment with *N*-nitrosobis(2-oxopropyl) amine (BOP), was evaluated using electron microscopy and immunohistochemistry. Electron microscopical examination of pseudoductular lesions, present in hamster pancreas 2–4 mo after treatment with BOP, demonstrated that acinar cells forming part of these lesions frequently lose their zymogen granules. However, convincing evidence of dedifferentiation of acinar cells to proliferating ductal/ductular cells was not found. Most ductal/ductular cells of the BOP-induced pseudoductular lesions stained positively with cytokeratins specific to ductal/ductular cells. Acinar cells were all negative and, moreover, those lining the pseudoductular lesions were frequently surrounded by cytoplasmic processes of adjacent cells that stained strongly positive with the cytokeratin antibody.

The present findings indicate that the early pseudoductular lesions, induced in exocrine pancreas of hamsters by BOP, originate from proliferating ductal/ductular rather than proliferating dedifferentiated acinar cells.

Key Words: Syrian hamster; preneoplastic lesions; exocrine pancreas; histogenesis; electron microscopy; immunohistochemistry.

INTRODUCTION

By far the greatest number of human adenocarcinomas of the exocrine pancreas are of the ductal type and only a small percentage show an acinar

*Author to whom all correspondence and reprint requests should be addressed.

cell differentiation (*1*). Rats and hamsters treated with azaserine or propylated nitrosamines, respectively, are the most frequently used experimental models to study pancreatic carcinogenesis (*2,3*). These two animal models markedly differ with respect to the histomorphology of the induced pancreatic tumors, being of ductal/ductular cell type in the hamster and acinar cell type in the rat (*2,4,5*). Because of the similarity of the induced tumors to those occurring in humans, the hamster model has been considered to provide a unique opportunity to study pancreatic carcinogenesis (*6*). The histogenesis of the ductal/ductular adenocarcinomas induced in hamsters, however, is a topic of debate in the literature. In BOP-treated hamsters, many tumors were found to develop within or in the vicinity of islets, in the form of "intra-insular ductules" associated with newly formed endocrine cells (nesidioblastosis).

Therefore, Pour et al. postulated that ductular and islet precursor cells are the foundation of the "pseudoductular lesions" and, hence, the adenocarcinomas (*5,6*). Recently, the centroacinar cell origin has been emphasized (*7,8*). Electron microscopical examination of pancreatic tumors, induced in hamsters by nitrosamines, has led to the conclusion that the tumors arise from existing ducts without any involvement of acinar cells (*8–10*). Other workers, however, claim that pancreatic adenocarcinomas in hamsters develop from acinar cells by dedifferentiation (*11–15*).

The differences in view on the histogenesis of pancreatic ductal/ductular adenocarcinomas, induced in hamsters by propylated nitrosamines, concentrate on two key questions: 1. "Are early pseudoductular lesions formed by proliferating dedifferentiated acinar cells or by proliferating ductal/ductular cells?" and 2. "Which category of the early, putative precancerous lesions is linked to a high progression probability and, hence, most relevant for the formation of pancreatic cancer?" The purpose of the present study was to find additional information to answer the first question by means of electron microscopic as well as immunohistochemical techniques.

MATERIALS AND METHODS

Seventy-eight male weanling Syrian golden hamsters were obtained from Charles River, Montreal, Canada. They were kept on softwood bedding in macrolon cages, five animals per cage under standard laboratory conditions. The animals were fed a semipurified diet the composition of which has been given in a previous paper (*16*). Seventy-two hamsters were injected subcutaneously (SC), once weekly with 20 mg BOP/kg body wt at 5, 6, and 7 wk of age. Six animals served as untreated controls. BOP (Ash Stevens, Inc., 5861 John C. Lodge Freeway, Detroit, MI) was dissolved in 0.9% NaCl solution immediately before use. Twenty-six animals, including two untreated controls, were sacrificed 2, 3, or 4 mo after the last treatment with the carcinogen.

The animals were anesthesized by ether, exsanguinated by cannulating the abdominal aorta, and then examined for gross pathological changes. The entire pancreas and liver from each animal were excised and weighed. At each interval, part of the pancreata from 12 BOP-treated animals and one untreated control were processed for electron microscopy. For this purpose, the tissue

was fixed for 20 h in 2% paraformaldehyde with 1% glutaraldehyde in 0.1*M* cacodylate buffer (pH 7.35, 900 mOsm) and stored in 2% paraformaldehyde in 0.1*M* cacodylate buffer (pH 7.35, 820 mOsm). Graded aceton–water mixtures were used for dehydration. An Epon–Araldite mixture was used for embedding. Semi-thin sections, stained with 1% toluidine blue in 1% borax solution, and ultra-thin sections, contrasted with magnesium uranyl acetate and lead citrate, were cut on a Reighert Ultracut E and examined with a Zeiss photomicroscope and a Philips EM410LS electron microscope at 60 kV. The remaining parts of the pancreata were fixed in 10% buffered formalin and processed for microscopy by conventional methods, step-sectioned at 4 μm, and stained with hematoxylin and eosin (H&E).

The pancreata of the 12 remaining BOP-treated animals and one untreated control killed at each time interval were fixed for 18 h in formaldehyde sublimate and subsequently processed in paraffin for immunohistochemical, as well as for routine (H&E), light microscopy. Sections 4 μm thick were mounted on glass slides and dried.

The polyclonal antiserum to cytokeratin filaments contained subunits against 48, 51, 52, 56, 58, and 60 kD, as previously described (*17,18*), and differentiated ductal, ductular, and centroacinar cells from acinar cells in the hamster pancreas. Specific antiserum to hamster acinar cells was a monoclonal antibody raised in mice, as reported by Parsa et al. (*19*). All aforementioned antibodies were obtained from Dakopatts a/s, Glostrup, Denmark, except for the antibody against hamster acinar cells, kindly provided by I. Parsa.

After deparaffination, mercury deposits were removed by lugol, and the slides were bleached with sodium thiosulphate. Subsequently, endogenous peroxidase was blocked by 0.3% hydrogen peroxide in methanol. Thereafter, the slides were washed in phosphate-buffered saline (PBS, 0.01*M*, pH 7.4). The cytokeratin antigen was demonstrated by the peroxidase–anti-peroxidase method and the acinar cell antigen was demonstrated by an indirect method (*20*). The specificity of the cytokeratin reaction was verified by absorption with purified keratin and by replacing the specific antiserum with PBS.

RESULTS

At each sacrifice, the pancreata of the hamsters showed pseudoductular lesions lined by flattened, cuboidal, or columnar epithelium. The incidence and size of the pseudoductular lesions demonstrated an increase in time resulting in the presence of more lesions with a greater diameter at 4 mo than at 2 mo posttreatment. However, at each sacrifice, the size of the various pseudoductular lesions observed varied considerably. Qualitatively, the pseudoductular lesions did not reveal striking differences at the three intervals except after 2 mo relatively more acinar cells appeared to form part of the pseudoductular lesions than after 4 mo.

In normal hamster pancreas, strong specific positivity was seen in ductal, ductular, and centroacinar cells. Much fainter staining was seen in the pancreatic islets. No staining at all was observed in normal acinar cells. The ductal/ductular cells lining the pseudoductular lesions stained positively with the

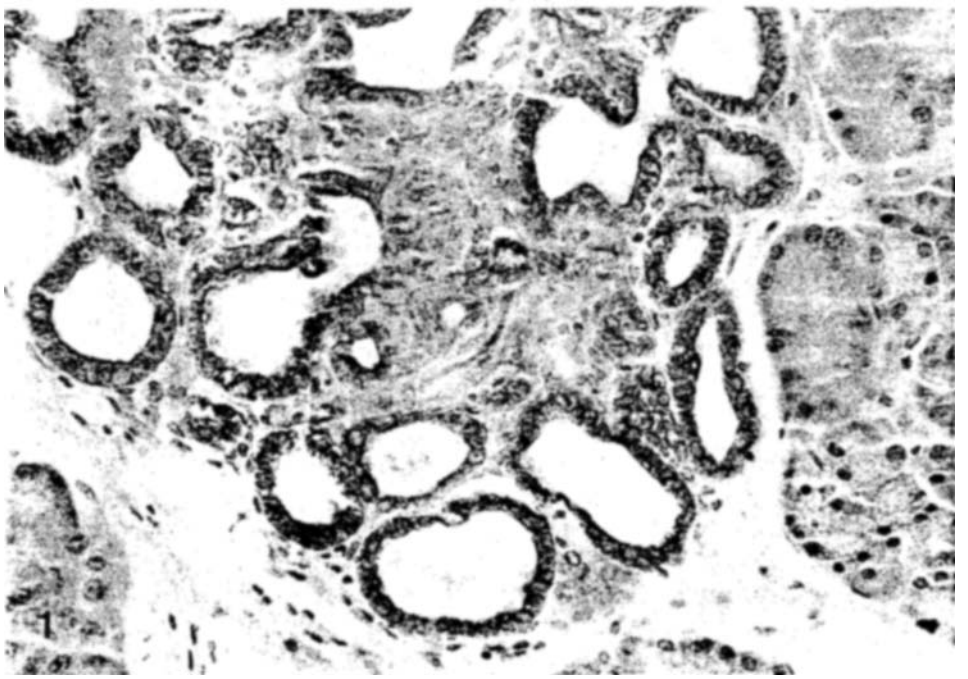

Fig. 1. Pseudoductular lesion lined by cuboidal eplithelium in hamster pancreas 4 mo after treatment with BOP. Immunoperoxidase stain with antibody to cytokeratin. Note the strong positive staining of the cuboidal pseudoductular cells (×350).

cytokeratin antibody (Fig. 1). The acinar cells forming part of the pseudoductular lesions could be easily recognized by their zymogen granules; they did not stain at all with the cytokeratin antibody (Figs. 2A,B). When applying an antibody specific to hamster acinar cells, the pseudoductular lesions frequently appeared to be lined by one or more acinar cells that occasionally were seen to be expelled into the lumen of the pseudoductule (Fig. 3). This "pushing away" of acinar cells was also observed with electron microscopy (Fig. 4).

Two months after the last injection of BOP, electron microscopical examination of the exocrine pancreas revealed several acinar cells with dilatation of the rough endoplasmic reticulum and marked mitochondrial swelling. At subsequent sacrifices, distension of the luminae of some acini and loss of zymogens in several acinar cells became more prominent. Occasionally, autophagic vacuoles packed with zymogen granules were seen. The luminal surface of the pseudoductular lesions were frequently covered by cytoplasmic processes of adjacent centroacinar cells (Figs. 5A,B and 6A,B), which were stained with the polyclonal cytokeratin antiserum (Figs. 2A,B).

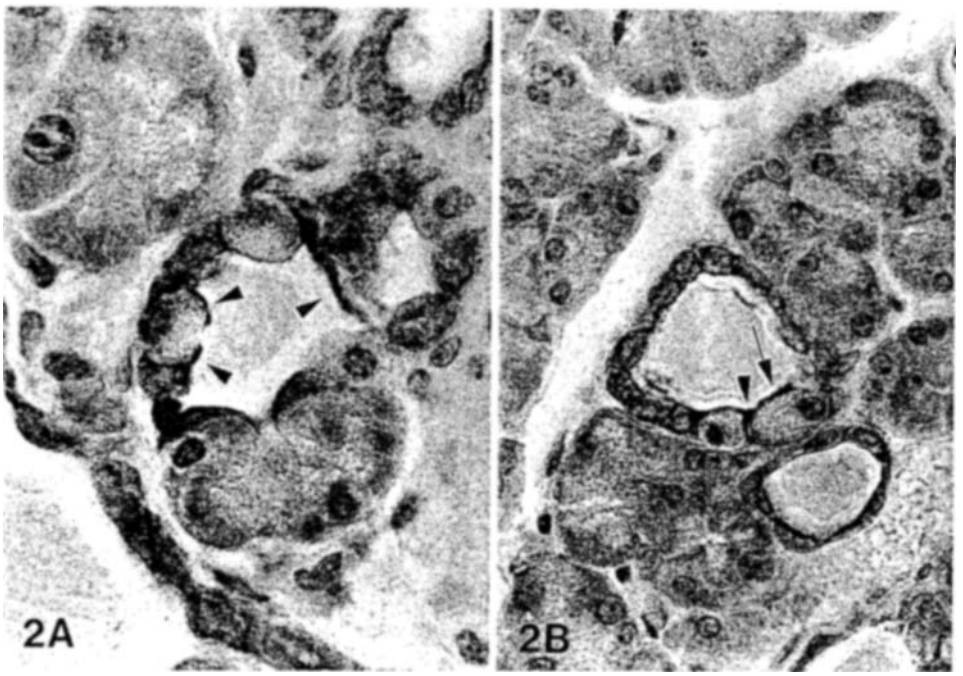

Fig. 2. (A) Acinar cells forming part of a pseudoductular lesion in hamster pancreas 4 mo after treatment with BOP. Immunoperoxidase stain with antibody to cytokeratin. Pseudoductular cells are clearly positive, whereas the acinar cells are negative. Note the cytokeratin-positive cytoplasmic processes of the adjacent pseudoductular cells (arrowheads) on the luminal surface of the acinar cell (×875). (B) Pancreas of a hamster 3 mo after treatment with BOP. Immunoperoxidase staining with antibody to cytokeratin. Cytokeratin-positive pseudoductular cell between two negative acinar cells (arrowhead). Note the pseudoductular cell growing over the luminal surface of the neighboring acinar cell (arrow) (×875).

DISCUSSION

Several workers have postulated that not only ductal/ductular cells, but also acinar cells, may play an important role in the development of early pseudoductular lesions and ultimate ductular adenocarcinomas in BOP-treated hamsters (*14,15,21*). According to Flaks, the development of ductular adenocarcinomas comprises two steps: dedifferentiation of the acinar cells to duct-like cells, followed by proliferation of these dedifferentiated acinar cells.

It is difficult to understand, however, how cells that can hardly be distinguished from ductular or centroacinar cells can be classified with certainty as former acinar cells. The main characteristics of the acinar cells are the pres-

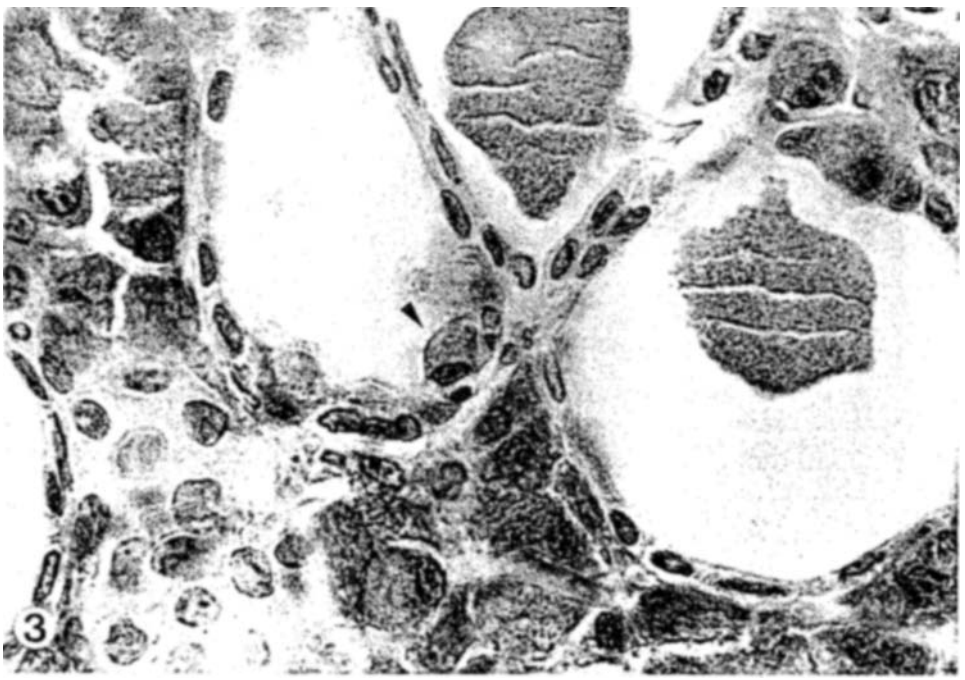

Fig. 3. Acinar cells within pseudoductular lesions in hamster pancreas 4 mo after BOP treatment. Immunoperoxidase stain with antibody to acinar cells. Note the unstained pseudoductular cells situated around the beneath the acinar cell giving the impression of the acinar cell being pushed into the lumen (arrowhead) (×875).

ence of abundant rough endoplasmic reticulum and zymogen granules. Cells with loss of zymogens could be considered either as transitional cells between acinar and ductal/ductular cells, or degenerating cells. However, since these cells did not show a typical ductal cytokeratin pattern or signs of proliferating activity, their participation in the development of pseudoductules is rather speculative. Loss of zymogen granules, whether or not by selective autophagy, and dilatation of rough endoplasmic reticulum have been interpreted as signs of a dedifferentiation process to which acinar cells of BOP-treated hamster pancreas are thought to be subjected (*14,15*). These features, however, are in no way indicative of a proliferative process and can also be found in cells subjected to various toxic substances (*22*).

The changes seen in the acinar cells are, in our view, indicative for a toxic effect exerted by the carcinogen. It is not illogical to assume that the loss of acinar cells results in proliferation of centroacinar cells. Using a multi-injection protocol, the number of acinar cells, as well as centroacinar cells, affected by the cytotoxic or carcinogenic action of BOP will be increased. We observed that acinar cells, being part of pseudoductular lesions, are either pushed away into the lumen of the pseudoductule or completely surrounded by processes of adjacent centroacinar cells.

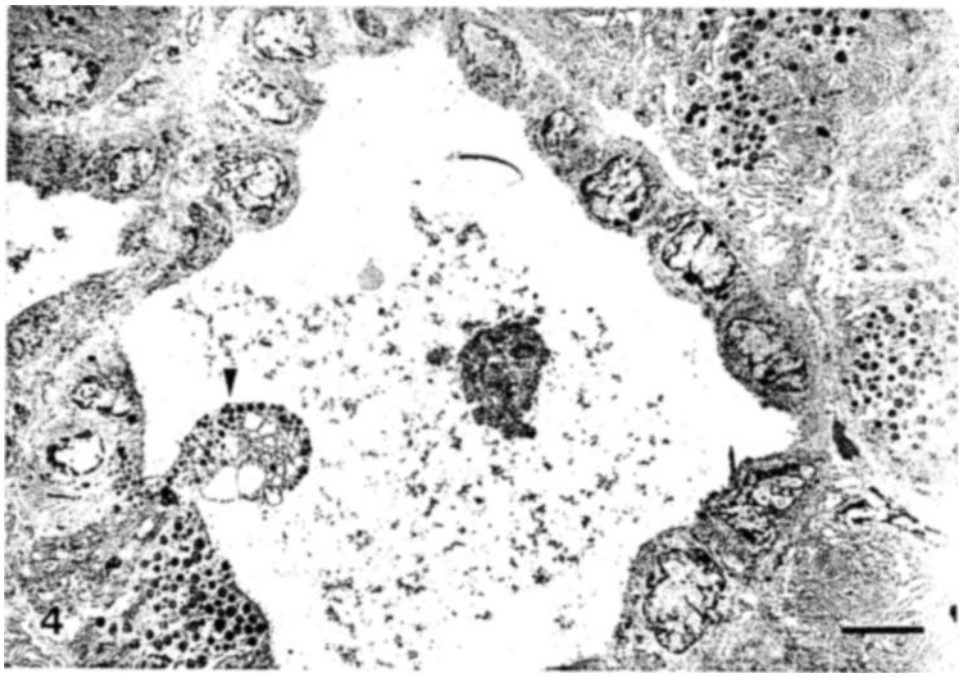

Fig. 4. Pancreas of a hamster 2 mo after treatment with BOP. The acinar cell has been expelled into the lumen of a pseudoductular lesion (arrowhead). Note the dilated rough endoplasmic reticulum. Bar = 2μm.

Our electron microscopical observations are in accordance with those of Pour et al. (*5,7,8,24*), who also described formation of cytoplasmic processes of centroacinar cells surrounding the acinar cells, leading to the isolation of acinar cells from the lumen and subsequent degeneration. We did not find any evidence for dedifferentiation of acinar cells to proliferating duct-like cells. Essentially, our findings are comparable with those of Willemer et al. (*23*) who induced pancreatitis in rats by cerulein and oleic acid and found that acinar cells, forming part of pseudoductules or tubular complexes, represent degenerating acinar cells that have no regenerative potency and lost their secretory and membrane characteristics (*23*). Therefore, it may be concluded from the present findings that the early pseudoductular lesions found in hamster pancreas 2, 3, and 4 mo after treatment with BOP originate from proliferating ductal/ductular cells rather than from dedifferentiated acinar cells. On the other hand, it is possible that degeneration of the acinar cells caused by the toxic effect of BOP plays an important role, if not a *"conditio sine qua non,"* in the carcinogenesis process. Bell and Ray (*25*) found that BOP-induced ductular adenocarcinomas in exocrine pancreas of hamsters stained positive with the cytokeratin antibody. It is tempting to combine their findings with those presented in this paper and those reported by Pour et al. (*8,24*) and to conclude that proliferating centroacinar cells are mainly

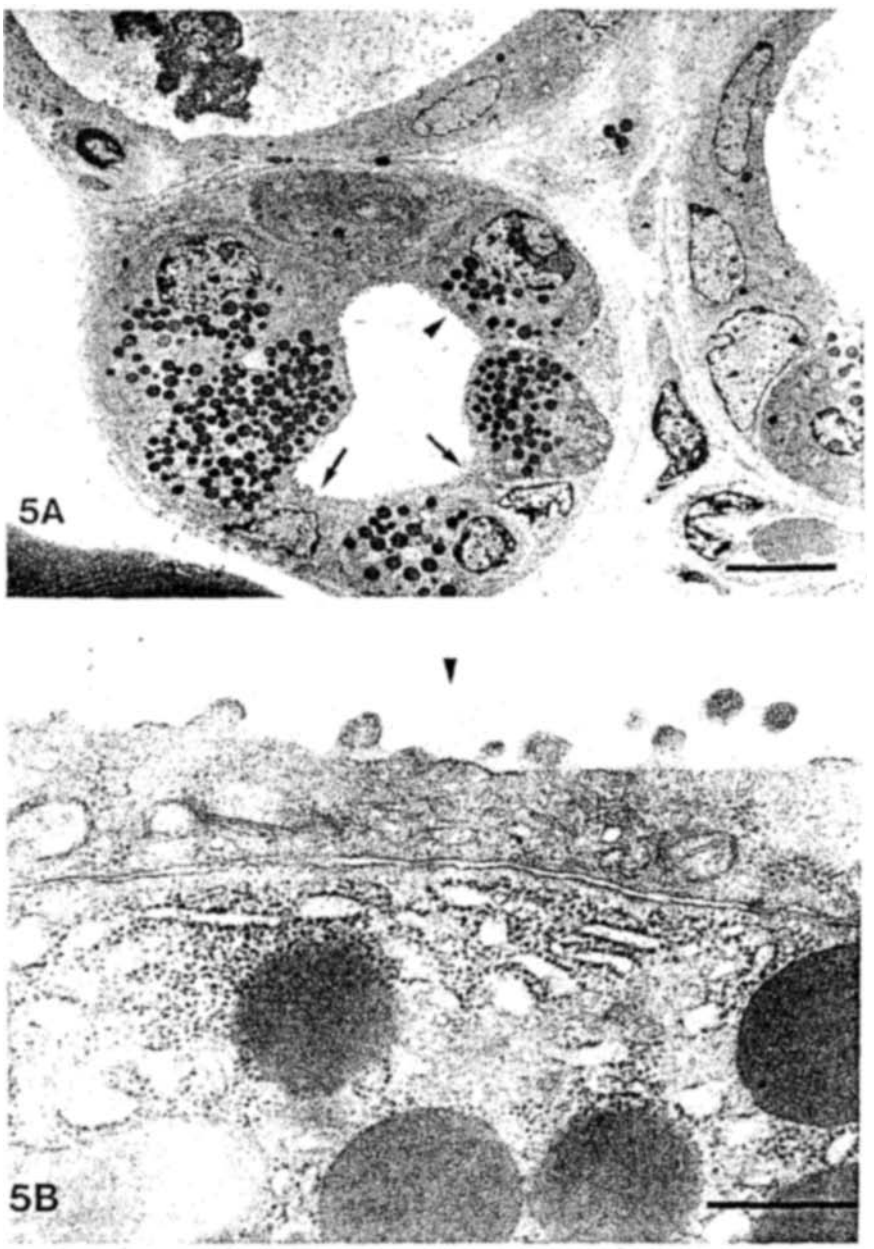

Fig. 5. (A) Pseudoductular lesion containing acinar cells in hamster pancreas, 4 mo after treatment with BOP. One acinar cell showing a strong reduction of zymogen granules is completely covered on the luminal surface by cytoplasmic processes of adjacent centroacinar cell(s) (arrowhead). Note the (enlarged) centroacinar cells adjacent to the acinar cells (arrows). Bar = 5μm. (B) Detail of Fig. 1. The cytoplasmic processes covering the acinar cell is part of the cell that lacks much endoplasmic reticulum and zymogen granules, indicating a ductal/ductular rather than an acinar origin. Bar = 0.5μm.

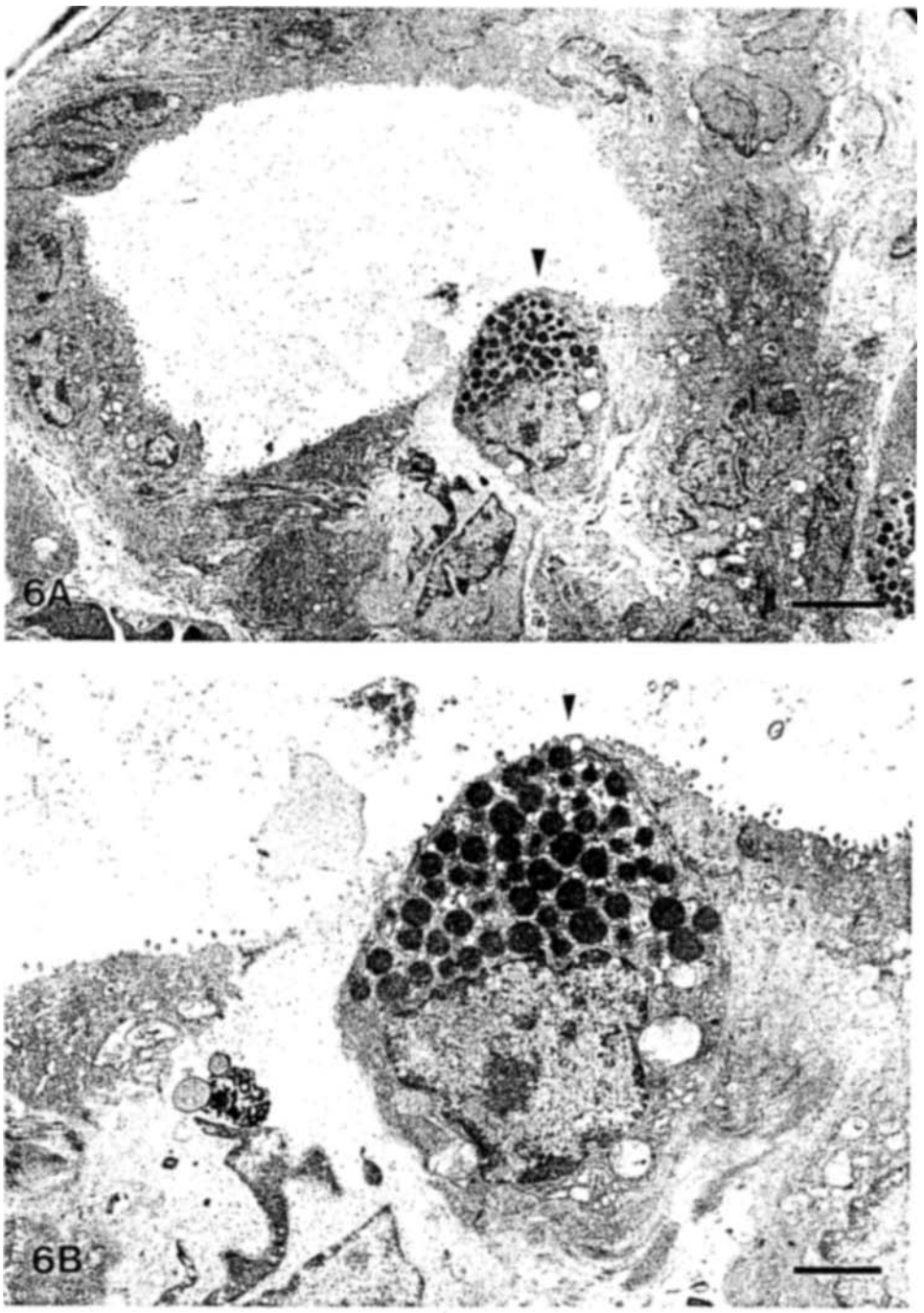

Fig. 6. (A) Hamster pancreas 3 mo after treatment with BOP showing a pseudoductular lesion containing one acinar cell. Note the cytoplasmic process of the adjacent centroacinar cell on the luminal surface (arrowhead). Bar = 5μm. (B) Detail of Fig. 2A. Note the swollen mitochondria. Bar = 2μm.

involved in the formation of pseudoductular lesions and, hence, in the development of ductular adenocarcinomas in the exocrine pancreas of hamsters treated with BOP or its analogs. However, the relationship between the various types of early putative preneoplastic pseudoductular lesions and the ultimate ductular adenocarcinomas is not fully elucidated and needs to be further evaluated.

ACKNOWLEDGMENTS

We wish to thank A. van Garderen-Hoetmer, P. M. Scherrenberg, A. A. van Tuyl, M. G. J. Nederhoff, and M. Stoepker for biotechnical assistance. We are grateful to V. J. Feron and R. J. J. Hermus for evaluation of the draft. This work was supported by grant CIVO 84-1 from the Koningin Wilhelmina Fonds (The Netherlands Cancer Foundation).

REFERENCES

1 Cubilla AL, Fitzgerald PJ. Surgical pathology of tumors of the exocrine pancreas. Moossa AR, Ed. Tumors of the Pancreas, Williams and Wilkins, Baltimore, 1980; 159–218.
2 Longnecker DS. Experimental models of exocrine pancreatic tumors. Go V, Dimagno E, Lebanthal E, Brooks F, Gardner J, Scheele G, Eds. The Exocrine Pancreas, Biology, Pathobiology and Diseases, Raven Press, New York, 1986; 443–458.
3 Longnecker DS, Wiebkin P, Schaeffer BK, Roebuck BD. Experimental carcinogenesis in the pancreas. Int. Rev. Exp. Pathol. 1984; 26: 177–229.
4 Scarpelli DG, Rao MS, Reddy JK. Studies of pancreatic carcinogenesis in different animal models. Environ. Health Perspect. 1984; 56: 219–227.
5 Pour PM, Wilson RB. Experimental tumors of the pancreas. Moossa AR, Ed. Tumors of the Pancreas, Williams and Wilkins, Baltimore, 1980; 37–158.
6 Pour PM, Runge RG, Birt D, Gingell R, Lawson T, Nagel D, Wallcave L, Salmasi SZ. Current knowledge of pancreatic carcinogenesis in the hamster and its relevance to human disease. Cancer 1981; 47: 1573–1587.
7 Pour PM. Histogenesis of exocrine pancreatic cancer in the hamster model. Environ. Health Perspect. 1984; 56: 229–243.
8 Pour PM. Mechanism of pseudoductular (tubular) formation during pancreatic carcinogenesis in the hamster model. An electron-microscopic and immunohistochemical study. Am. J. Pathol. 1988; 130: 335–344.
9 Levitt MH, Harris CC, Squire R, Springer S, Wenk M, Mollelo C, Thomas D, Kingsbury E, Newkirk C. Experimental pancreatic carcinogenesis. Am. J. Pathol. 1977; 88: 5–28.
10 Takahashi M, Arai H, Kokubo T, Furukawa F, Kurata Y, Ito N. An ultrastructural study of precancerous and cancerous lesions of the pancreas in Syrian golden hamsters induced by BOP. Gann 1980; 71: 825–831.
11 Flaks B, Moore MA, Flaks A. Ultrastructural analysis of pancreatic carcinogenesis III. Multifocal cystic lesions induced by N-nitroso-bis(2-hydroxypropyl)amine in the hamster exocrine pancreas. Carcinogenesis 1980; 1: 693–706.
12 Flaks B, Moore MA, Flaks A. Ultrastructural analysis of pancreatic carcinogenesis IV. Pseudoductular transformation of acini in the hamster pancreas during N-nitroso-bis (2-hydroxypropyl)amine carcinogenesis. Carcinogenesis 1981; 2: 1241–1253.
13 Flaks B, Moore MA, Flaks A. Ultrastructural analysis of pancreatic carcinogenesis V. Changes in differentiation of acinar cells during chronic treatment with N-nitroso-bis (2-hydroxypropyl)amine. Carcinogenesis 1982; 3: 485–498.
14 Flaks B. Histogenesis of pancreatic carcinogenesis in the hamster. Ultrastructural evidence. Environ. Health Perspect. 1984; 56: 187–203.

15 Carre-Llopis A, Loridon-Rosa B, Escribano MJ. Ultrastructural changes in acinar cells of hamster pancreas in chemically induced carcinogenesis. Cell Biology Int. Rep. 1987; 11: 665–669.
16 Woutersen RA, Garderen-Hoetmer van A, Bax J, Feringa AW, Scherer E. Modulation of putative preneoplastic foci in exocrine pancreas of rats and hamsters. I. Interaction of dietary fat and ethanol. Carcinogenesis 1986; 7: 1587–1593.
17 Steinert PM. The extraction and characterization of bovine epidermal alpha-keratin. Biochem. J. 1975; 149: 39–48.
18 Franke WW, Weber K, Osborn M, Schmid E, Freudenstein C. Antibody to prekeratin. Exp. Cell Res. 1978; 116: 429–445.
19 Parsa I, Marsh WH, Wong JY, Cleary CM. Hamster pancreas acinar cell post-differentiation antigen, detected by murine monoclonal antibody. Int. J. Pancreatol. 1986; 71–81.
20 Polak JM, Noorden van S. Immunocytochemistry, practical applications in pathology and biology. Wright PSG, Bristol, 1983.
21 Woutersen RA, Garderen-Hoetmer van A, Longnecker DS. Characterization of a 4-month protocol for the quantitation of BOP-induced lesions in hamster pancreas and its application in studying the effect of dietary fat. Carcinogenesis 1987; 8: 833–837.
22 Constantinides P. Ultrastructural Pathobiology, Elsevier, Amsterdam, 1984.
23 Willemer S, Elsasser HP, Kern HF, Adler G. Tubular complexes in cerulein- and oleic acid-induced pancreatitis in rats: Glycoconjugate pattern, immunohistochemical, and ultrastructural findings. Pancreas 1987; 2: 669–675.
24 Pour PM, Parsa I, Hauser H. Evidence for partial exocrine acinar differentiation in experimentally induced pancreatic ductal/ductular cell tumors. Int. J. Pancreatol. 1987; 1: 47–58.
25 Bell RH Jr, Ray MB. (Short Communication), Cytokeratin antigen in BOP-induced pancreatic tumors—implications for histogenesis. Carcinogenesis 1987; 8: 1563–1566.