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THREE-DIMENSIONAL RECONSTRUCTION OF PERINEURAL INVASION IN CARCINOMA OF THE EXTRAHEPATIC BILE DUCTS

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SUMMARY

Three-dimensional (3D) reconstruction can be used to study the structural relationships between various tissue components and can help in the understanding of disease processes. Examples of perineural invasion have been reconstructed in two cases of adenocarcinoma of the extrahepatic bile duct. Ninety-six serial 5 μm sections were taken from both cases stored on file and 3D images were constructed using a Kontron VIDAS image analysis system. Both cases showed continuity of tumour cells within the perineural space. The isolated islands of malignant glands seen by conventional microscopy were shown to be in continuity with larger tumour cell masses via a complex branching network. In addition, direct continuity was demonstrated between malignant glands within the perineural space and those within the surrounding stromal tissue. During growth, the tumour appeared to have followed the plane of least resistance, although the availability of the perineural space may itself have been shaped by pressure effects and/or proteolytic enzyme secretion. Three-dimensional reconstruction of perineural invasion in adenocarcinoma of the extrahepatic bile ducts shows the value of this technique in demonstrating the structural relationships between the tumour and the host nerve bundle.

KEY WORDS—**perineural invasion; bile duct carcinoma**

INTRODUCTION

The prognosis of carcinoma of the extrahepatic bile ducts remains relatively poor despite improvements in diagnosis, treatment, and the identification of parameters such as histological grade, lymph node metastasis, and perineural invasion.[1,2]

The spread of malignancy in such cases is thought to be through infiltration of the stroma, venous infiltration, and perineural invasion.[3–6] The structural relationship between the invading tumour and the peripheral nerves appears to be an important means of tumour spread. It has been suggested that perineural invasion is one possible mode of entry for tumours to the lymphatic system, although Larson *et al*[4] found no evidence of lymphatic channels associated with peripheral nerves.

Computer-assisted three-dimensional (3D) reconstruction of tissue components can help in the understanding of disease processes.[6–11] We have looked, therefore, at the possible use of computerized 3D reconstruction in studying the structural relationships between the host nerve bundle and the invading tumour.

MATERIALS AND METHODS

Histology

Paraffin wax-embedded blocks from two cases of carcinoma of the extrahepatic bile duct were taken from file. Case 1 was of a 51-year-old male and case 2 of a 62-year-old female. Ninety-six serial sections were cut at 5 μm and dried overnight at 55°C. Structural distortions were minimized by adopting specific handling parameters. Sectioning artefact was reduced by cooling the tissue block with ice prior to sectioning. The temperature of the water bath was also controlled.

In order to highlight the malignant glands and to test the ability of the imaging system to resolve them, the first case was stained for carcinoembryonic antigen (CEA) using a polyclonal antiserum (Dako) diluted 1:500 and absorbed with human liver powder.[12] Immunoreactivity was localized using a standard peroxidase StreptAvidin biotin method (Dako) and hydrogen peroxide–diaminobenzidine as the chromogen. Sections from the second case were stained by a conventional haematoxylin and eosin (H&E) stain.

3D reconstruction

After histological assessment, images of nerve and tumour from sequential sections were acquired using a Sony single chip CCD camera and digitally stored as 256 × 256 pixel black and white images. A Kontron VIDAS image analyser (Imaging Associates, Cambridge) was used to capture images, which were then stored on a DPL Optistore 650 Mbyte optical disk drive.

Alignment of sections was achieved by camera rotation and was based on the nearest match of the major structural features on sequential sections.

Each image was recalled from disk. The nerve perimeter and basement membrane of the malignant glands were traced using a digital cursor overlying the image. Data for the basement membrane and nerve perimeter were registered in different channels.

The stored profiles from each section were digitally stacked to produce a reconstructed object which could

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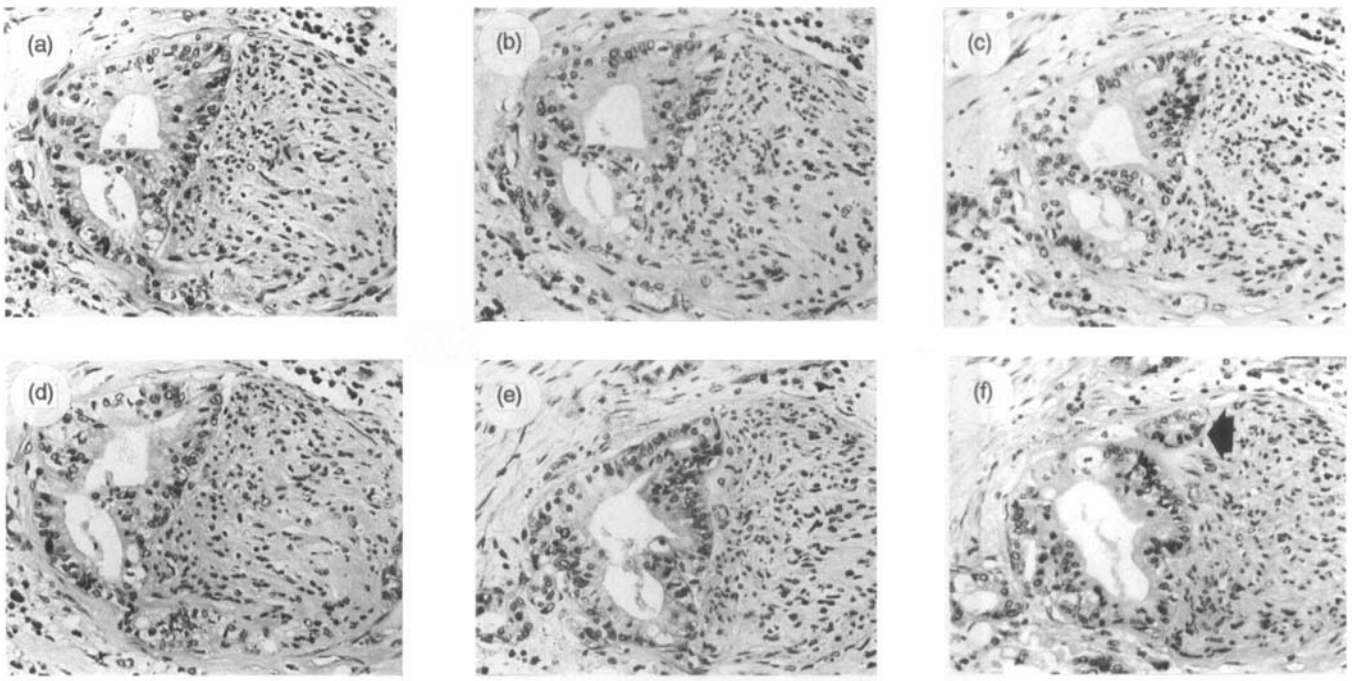

Fig. 1—(a–f) Serial sections of perineural invasion showing the development of malignant glands. The two glands in a and b are branches of the single gland seen in subsequent sections. 3D reconstruction helps us to appreciate the relationship of the isolated gland (f, small arrow), showing it as a branch from the main malignant gland

be viewed on the computer screen. This was carried out using the Kontron 3D reconstruction software for the VIDAS image analyser. The 3D images could then be stored on disk.

Reconstructed images could be viewed on the computer screen at any angle by varying the rotation, tilt, and elevation angles set in the software. By use of different channels previously selected for the glands and nerve, structural features could be viewed individually or simultaneously.

RESULTS

Both cases were confirmed as showing apical CEA positivity in well-differentiated glands involved in perineural invasion. Associated normal glands were uniformly CEA-negative.[12] Images of the sections were easily transferred to the monitor and used in reconstruction, irrespective of the staining method.

Following digital stacking (Figs 1a–1f), the profile of each section was used to produce a reconstructed object which could be viewed on the computer screen (Figs 2 and 3).

Using computerized 3D reconstruction, we found that both cases showed continuous and extensive branching by the malignant glands within the respective perineural spaces. Some branches had grown laterally within the perineural space whilst others followed the long axis plane of the nerve. These branches demonstrated the close association between the tumour and the host nerve bundle, with the tumour closely following the orientation of the nerve. When this changed, it was followed by the tumour. Some branches of tumour cells became entrapped within the nerve bundle itself, appearing as islands of malignant cells on conventional microscopy (Figs 1a–1f). Those branches of tumour remaining within the perineural space showed a similar continuity with malignant glands within the bile duct stroma (Fig. 3).

Whilst it was difficult to reconstruct all the lumina of the malignant glands found in the two cases studied, those which were followed formed a connected and united network.

DISCUSSION

Perineural invasion by malignant glands in carcinoma of the extrahepatic bile ducts is an important feature of malignant disease,[1] with a reported incidence varying from 29 to 78 per cent.[2,3,13] This is the first study to use 3D reconstruction to examine perineural invasion of the extrahepatic bile ducts. This was carried out to help identify the structural relationships between the invading tumour and peripheral nerve bundles and thus help in the understanding of tumour growth and spread.[8–10]

To aid growth and development, invading neoplastic cells of many tumours synthesize their own extracellular matrix (ECM) proteins, providing a suitable microenvironment and basement membrane.[14] The basement membrane influences the cell shape and expression of adhesion molecules, ultimately affecting the gene products of the cell.[15–17] Laminin has been described as being synthesized by both benign and malignant bile duct cells.[18] We used the basement membrane of well-differentiated adenocarcinoma in this study to aid the orientation of major structural features for 3D reconstruction.

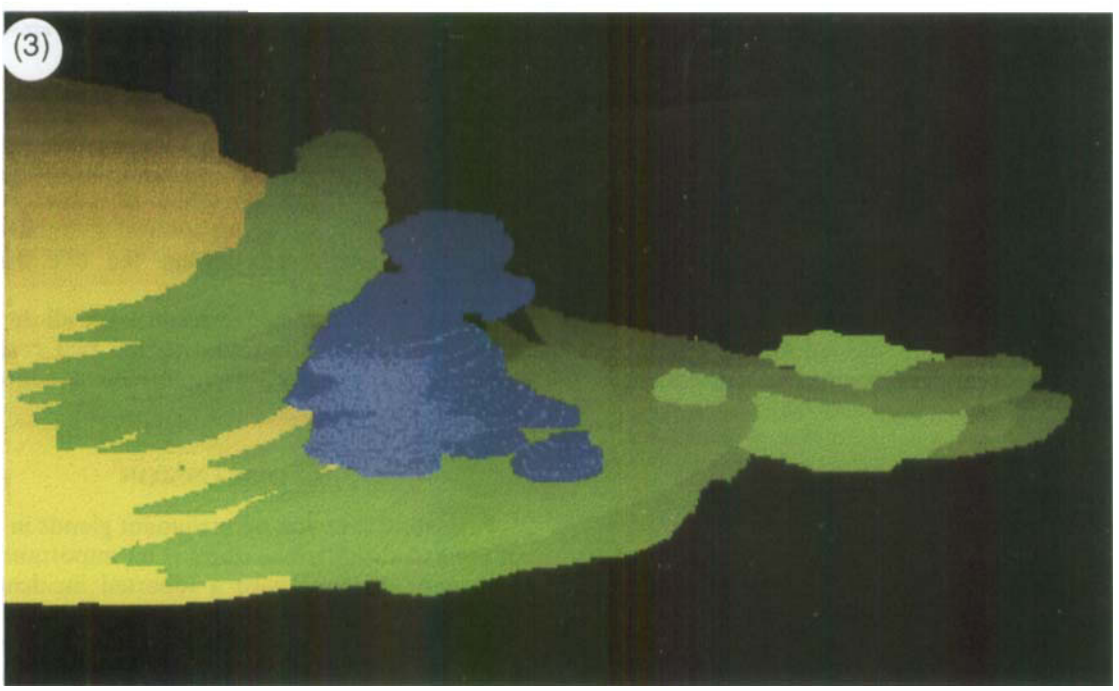

Fig. 2—3D reconstruction of a well-differentiated adenocarcinoma (green) surrounding a nerve bundle (yellow). The image is tilted and rotated to demonstrate how the tumour has invaded into the nerve bundle (arrow), appearing as an island of tumour within the nerve. The approximate height of the nerve segment is 250 μm

Fig. 3—3D reconstruction of perineural invasion in which the well-differentiated adenocarcinoma (green) follows and surrounds the contour of the nerve bundle (yellow), whose long axis runs from left to right. The image has been tilted and rotated to demonstrate how the tumour branches within the perineural space (green) and is continuous with the malignant glands within the stroma (blue). The height of the nerve segment is approximately 100 μm

The two cases studied here indicated continuity of tumour cells within the perineural space. What appear on conventional sections as isolated islands of malignant glands are shown to be in continuity with larger tumour cell masses, via a complex branching network. In addition, direct continuity was demonstrated between malignant glands within the perineural space and those within the surrounding stroma, although more cases would need to be examined to confirm this.

Malignant cells arising from extrahepatic bile ducts metastasize via stromal infiltration, penetrating the serosal surface to reach surrounding tissues.[5,19] Some authors see tumour growth within the perineural space as indicating that local tumour spread follows the plane

of least resistance,[3,4] but this is difficult to asses on histological examination. It is not safe to assume that what appears least dense in a section necessarily correlates with least resistance. Due to the widespread branching of the tumour within the cases examined, however, the perineural space available for tumour growth may itself be influenced by the tumour and the shape of the nerve bundle may be altered by pressure effects and/or proteolytic enzymes to facilitate transmembrane invasion.[20]

The lumina of malignant glands have been followed in gastrointestinal neoplasia by Takahashi and Iwama.[6,7] Due to the plane of sectioning, the lumina of malignant glands seen in the present study were often incomplete and could not be used for reconstruction. Those which were followed formed a luminal network. At all levels, these gave the appearance of a united structure.

The time dedicated to completing the input of data was one afternoon per case. A greater amount of time was spent on the analysis of the 3D images, understanding the relationships of the structures involved and representing them in composite images. While the system allows eight image components to be viewed simultaneously, interpretation was difficult when more than three structures were viewed together, due to the blinding of components in the background by overlapping structures in the foreground. This often masked important structural features. Interpretation of the image was greatly enhanced by the ability to orientate the image by changing the angles of rotation, tilt, and elevation.

Three-dimensional reconstruction of perineural invasion in adenocarcinoma of the extrahepatic bile ducts shows the value of this technique in demonstrating structural relationships in malignant disease.

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