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Technical method

Image analysis—a quantitative technique for studying normal and diseased microvasculature

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Many different techniques have been used to study the normal and diseased microvascular anatomy of various organs.[1] The most popular method has usually been various forms of injection using Indian Ink,[2] gelatine and barium suspension,[3] and silicone compounds.[4] Problems with these techniques include leakage of injection media, poor control of injection with variable filling of vessels, and subjective interpretation of the results. Image analysis systems have provided objectivity and precision of measurement and are increasingly used in pathology. Image analysis has been used to study the microvasculature of the human lower oesophagus in normal subjects and in patients with portal hypertension. The methodology of this technique is reported.

Material and methods

IMAGE ANALYSIS SYSTEM

A Leitz TAS Plus image analysis system was used (Leitz, Wetzlar, West Germany). This is an automatic image analysis system, which works on the picture point principle using a hexagonal raster to give a digitalised image for analysis. The slide containing the section to be studied is placed on the automatic microscope stage (microscope magnification × 100); the image is scanned by a linear measuring 1¼″ Plumbicon camera tube and accurately produced on a high contrast, high resolution television monitor. The points of the electronic image are stored in six 256 × 256 high speed binary image memories. The monitor serves for manual instruction input using a light pen and the control computer employs a DEC RT-II operating system and uses TASIC language.

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STAIN TECHNIQUE

Initially the 5 μm sections of oesophagus were stained with haematoxylin and eosin. This was unsatisfactory, however, in that it was difficult to recognise the small vessels and to distinguish the muscularis mucosa and muscularis on the black and white image on the monitor.

Several combinations of stains were assessed, and the most satisfactory results were obtained with Weigert's resorcin fuchsin stain counterstained with Masson's ponceau-2R-acid fuchsin stain.[5] With the Weigert's stain the vessels are well outlined by the black staining of the internal elastic lamina. The counterstaining with the Masson's stain shows the muscle layers, including the muscularis mucosa, which appears dark red.

Intact specimens of oesophagus and stomach were obtained at necropsy. After primary fixation in 10% buffered formalin and post-fixation in mercuric formol, transverse sections were taken at 1 cm intervals from the lower end of the oesophagus. After embedding in paraffin, 5 μm sections were cut using a rotary microtome. The sections were treated with xylol to remove the paraffin wax and washed with methylated spirit. They were then treated with 0·75% iodine in 70% alcohol for 5 min. The iodine was removed with 2% sodium thiosulphate in 20% alcohol for 2 min and washed in running tap water for 5 min. The sections were then treated with 0·25% potassium permanganate for 10 min, rinsed in water, and placed in 5% oxalic acid for 20 min. After being washed with water and rinsed in methylated spirit, the sections were placed in Hart's elastic tissue stain for 24 h.

Excess stain was removed with methylated spirit, washed in water, and differentiated in acid alcohol. The sections were then placed in Masson's ponceau fuchsin (diluted 1/10 with distilled water) for 6 min. They were transferred into a 1% solution of phosphomolybdic acid and left until the connective tissue lost all traces of ponceau fuchsin. After dehydration in absolute alcohol the sections were cleared in xylene and mounted in Canada balsam. This combined technique produced black staining of the elastic tissue giving good delineation of vessels, and the muscle layers were also clearly shown on the television monitor.

Ten fields were chosen from each slide using a system of random numbers. The light pen was used to trace round the area of lamina propria and adjacent submucosa of each field, and the areas of vessels were determined by tracing round their outlines. A computer programme was written which calculated the number of vessels in the lamina prop-

ria, mean vessel area, and relative area occupied by the vessels within the total area of lamina propria measured. Similar data were obtained for the adjacent submucosa. Results were expressed in μm.[2] Only vessels greater than 20 μm in diameter were assessed.

Results

NORMAL SPECIMENS (n = 20)

The relative mean area occupied by vessels in the oesophagus 2 cm from the oesophagogastric junction was 19·54 ± 6·9% in the lamina propria and 6·3 ± 3·9% in the submucosa. The average number of vessels in the lamina propria over 10 fields was 37·9 ± 7·9, and in the submucosa the corresponding figure was 17·7 ± 4·6. The mean vessel area was 3859·5 ± 4388·3 μm^2 in the lamina propria and 2442·9 ± 1688·4 μm^2 in the submucosa.

VARICES SPECIMENS (n = 5)

The oesophagi from five patients with varices and portal hypertension, were studied using the same method. The relative mean area occupied by vessels in the oesophagus 2 cm from the oesophagogastric junction in the five patients with varices was 27·1 ± 10·1% in the lamina propria and 8·6 ± 3·5% in the submucosa. The average number of vessels in the lamina propria over 10 fields was 19·8 ± 2·8 and in the submucosa the corresponding figure was 14·6 ± 2·7. The mean vessel area was 9534·4 ± 5037·6 μm^2 in the lamina propria and 4247·9 ± 1863·9 μm^2 in the submucosa.

The data from the normal specimens were compared with those from the variceal specimens using the Mann-Whitney U test. There was no significant difference in the relative area occupied by vessels in either the lamina propria or submucosa in the two groups ($p = 0·1182$, $p = 0·135$ respectively). The number of vessels in the lamina propria was greater in the control specimens compared with the varices specimens ($p = 0·0007$); the mean vessel area in the lamina propria was greater in the varices specimens ($p = 0·0145$). The number of vessels in the submucosa did not differ in the two groups ($p = 0·107$), but the mean vessel area in the submucosa was greater in the varices group ($p = 0·0488$). It is evident that in patients with oesophageal varices some of the vessels are greatly increased in size 2 cm above the oesophagogastric junction, but the numbers of vessels greater than 20 μm may be decreased owing to a local pressure effect.

Discussion

The problems of conventional techniques for studying abnormalities of the microvasculature of organs have limited their use in pathological studies. Although resin corrosion casting methods give useful information especially with scanning electron microscopy,[6–9] they destroy the surrounding soft tissues and interpretation is difficult and subjective. Small vessels down to capillary level can be filled with a medium of gelatin and barium,[3] but there are several problems associated with this technique. The extent of vascular penetration is governed by the viscosity of the mass, amount and nature of the added pigment, and to a lesser degree by the injection pressure. Solidification of the mass depends on the pH, amount of added formalin, temperature, and concentration of gelatin. These many variable factors make the technique tedious and prone to error. In addition, the interpretation of the distribution of vessels studied by this technique is subjective. Most of the injection techniques used have worked best in organs with a single supplying artery and few draining veins, such as the kidney or spleen.[10] The oesophagus has many supplying vessels and draining veins and hence injection techniques are unreliable with variable filling of vessels. There is also the problem of leakage if the organ is injected after removal from the body.

A more recent approach is the use of microspheres. These are about 15 μm in diameter and are labelled with radioactive chromium. They have been used widely for studying mucosal blood flow.[11 12] Because of their small size (7–20 μm), however, they pass through the larger vessels in the wall of the intestine[13] and are therefore of little use for studying vessels above capillary size.

Recently, the addition of image analysis computers has allowed semiautomatic and automatic recording,[14] and formulae are now available for calculating volumes and areas of sections.[15] Bradbury has recently assessed the currently available image analysis systems.[16]

Image analysis systems have been used to study the microvasculature of the brain in portasystemic encephalopathy[17] and age changes in the vascular architecture of the rat cerebral cortex.[18] The vascularity of oral neoplasms in hamsters has also been studied using a semiautomatic analysis system.[19] This approach to the study of vascular changes in various pathological conditions can be recommended as providing objective quantitative data which can be subjected to statistical analysis. It is particularly suitable in organs, such as the oesophagus, which do not have a single supplying vessel suitable for injection studies. The combination of stains used by us is suitable for other studies where the relation between vessels and muscle is to be investigated.

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Letters to the Editor

Necrotising granulomata in prostatic resection specimens—a sequel to previous operation

We read with interest the article by Dr Lee and Dr Shepherd.[1] We have observed similar appearances in two patients, aged 61 and 62 years, who had second transurethral prostatectomies 22 and 25 days respectively after their first operations. There was no clinical evidence of preceding urinary infection or tuberculosis.

In both cases there were granulomata in the second specimen, whereas they were absent from the first, which showed only benign nodular hyperplasia. The giant cells, most of which were of the foreign body type and occasionally contained pigmented particles or fibrinoid material, were situated close to necrotic areas, and there was also an infiltrate of lymphocytes, plasma cells, neutrophils, and eosinophils; the last were numerous in one patient. There was frequent squamous metaplasia of prostatic ducts. We did not find distinctive linear and non-linear changes, as did Dr Lee and Dr Shepherd.

We agree that these lesions are induced by operative trauma. The squamous metaplasia may be the result of ischaemia, but the latter is probably not directly related to the granulomatous reaction since granuloma is not a feature of spontaneous prostatic infarction.

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Anti-DNA immunoassay

Concern recently expressed[1] about the quality of anti-DNA test kits is well founded. Single stranded regions were present in all the samples of labelled native DNA from different commercial sources examined by Medoff.[2] Such defects increase binding values and decrease the specificity of the Farr test.

Since its introduction we have used repeatedly native ^{14}C DNA from Amersham in routine immunoassays, though both the stability and the molecular weight distribution of this material are unspecified. Of the many batches purchased, one, specially treated to remove single stranded regions, gave generally lower serum binding values, with some close to zero. As most batches appear to be incompletely double stranded we have reluctantly adopted the Farr test for antibodies to ss-DNA, using heat treated material, despite its disadvantages.

Though tests for antibody to native DNA may be valuable for prognosis and treatment, their diagnostic value is uncertain. Using phage derived native DNA Swaak *et al*[4] found increased values were given by sera from patients who did not satisfy the diagnostic criteria for systemic lupus erythematosus.

The Crithidia lucilae immunofluorescence test is deservedly popular, but the problems of variation between observers and in the composition of the labelled antiserum remain. It may also detect antiprotein antibodies in some circumstances.[5]

The stimulation of antibody production to native DNA may no longer be considered unique to systemic lupus