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Human colorectal cancers display abnormal Fourier-transform infrared spectra

(colon cancer/infrared spectroscopy/high-pressure spectroscopy/tumor markers)

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ABSTRACT **Fourier-transform infrared spectroscopy (FT-IR) was applied to the study of tissue sections of human colorectal cancer. Pairs of tissue samples from colorectal cancer and histologically normal mucosa 5–10 cm away from the tumor were obtained from 11 patients who underwent partial colectomy. All cancer specimens displayed abnormal spectra compared with the corresponding normal tissues. These changes involved the phosphate and C—O stretching bands, the CH stretch region, and the pressure dependence of the CH_2 bending and C=O stretching modes. Our findings indicate that in colonic malignant tissue, there are changes in the degree of hydrogen-bonding of (*i*) oxygen atoms of the backbone of nucleic acids (increased); (*ii*) OH groups of serine, tyrosine, and threonine residues (any or all of them) of cell proteins (decreased); and (*iii*) the C=O groups of the acyl chains of membrane lipids (increased). In addition, they indicate changes in the structure of proteins and membrane lipids (as judged by the changes in their ratio of methyl to methylene groups) and in the packing and the conformational structure of the methylene chains of membrane lipids. The cell(s) of the malignant colon tissues responsible for these spectral abnormalities is unknown. Cultured colon adenocarcinoma cell lines displayed similarly abnormal FT-IR spectra. The diagnostic potential of the observed changes is discussed.**

In recent years spectral methods have been used in the evaluation of malignancy, and some attempts have been made to utilize them as a diagnostic tool. Most of the work involves NMR spectroscopy (1–3); however, the initial enthusiasm for the diagnostic usefulness of NMR has subsided considerably (4).

Infrared spectroscopy is becoming an increasingly powerful tool for the study of the composition and structure of cellular components within intact tissues (5–7). Currently, methodological and technological advances are greatly enhancing the sensitivity of infrared spectroscopy. In particular, appreciation of the role of pressure on spectral parameters such as frequency, intensity, band shape, and band splitting and the consequent advances in high-pressure instrumentation have further facilitated the analysis of vibrational spectra (8–10).

In this paper we report our findings from the analysis of human colon cancer tissues by Fourier transform infrared (FT-IR) spectroscopy that was also combined with the use of high-pressure (pressure-tuning) infrared spectroscopy, when appropriate. Our data, showing clear-cut spectral differences between normal and malignant colonic tissue, suggest the potential applicability of this approach to a host of biological problems.

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PATIENTS AND METHODS

Patients. The 11 patients whose tissue samples were studied underwent partial large-bowel resection for colorectal cancer at North Shore University Hospital—Cornell University Medical College, Manhasset, NY. Five were women and 6 were men; their average age was 75 years (range, 59–84). Staging of tumors using the modified Dukes' classification (11) showed 1 patient with stage A tumor, 3 with stage B1, 5 with stage B2, and 2 with stage C.

Tissue samples were obtained immediately after bowel resection. From each patient, samples were obtained from both the tumor itself and the normal-appearing mucosa 5–10 cm away from the tumor, frozen in liquid nitrogen, and stored at −180°C until used. Each tissue sample was cut into several pairs of small segments (thickness, ≈1 mm; surface area, ≈1–2 mm^2). One member of each pair was used for spectroscopic studies. From the other member of the pair, 5-μm-thick microtome sections were cut from the side facing the companion tissue segment used in spectroscopy. These sections were fixed in formaldehyde, stained with hematoxylin and eosin, and examined histologically by two pathologists. The composition of each tissue section was scored blindly as a percentage of malignant and normal segments. In this way we were able to monitor as closely as possible the histological composition of each tissue section examined spectroscopically. Technical aspects of tissue sample collection for these studies are described elsewhere (12).

FT-IR Spectroscopy. Small amounts (typically 0.01 mg) of tissue samples were placed at room temperature, together with powdered α-quartz, in a 0.37-mm-diameter hole in a 0.23-mm-thick stainless steel gasket mounted on a diamond anvil cell. Pressures at the sample were determined from the 695-cm^{-1} infrared absorption band of α-quartz as described (13). FT-IR spectra at various pressures in kbars (1 bar = 100 kPa) were measured with a Digilab FTS-60 Fourier transform spectrometer using a liquid nitrogen-cooled mercury–cadmium–telluride detector. For each spectrum, 512 scans were coadded at a spectral resolution of 4 cm^{-1}. Data reduction was performed with software developed in our Ottawa laboratory.

Cell Lines. Human colon cell lines from the American Type Culture Collection were maintained in culture following standard protocols and the instructions of American Type Culture Collection. They included LoVo, HCT15, HT29, SW1116, SW403, SW480 (all colon adenocarcinomas) and CCD-18Co (normal human colon fibroblast). Cells were harvested from confluent plates either by trypsinization or gentle scraping of the tissue culture plates. Control experiments revealed that the infrared spectra of these cells were

Abbreviation: FT-IR, Fourier-transform infrared spectroscopy.

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not affected by the method of cell harvesting. Briefly, cells were washed with phosphate-buffered saline (PBS) and incubated with 3 ml of trypsin/EDTA (GIBCO and BRL) for 1 min at room temperature; after trypsin was aspirated, the cells were incubated at 37°C for 6 min. The detached cells were resuspended in 10 ml of PBS and washed once with PBS and aliquots were pelleted by centrifugation—all at 4°C. The pellet was stored in liquid nitrogen until analyzed by spectroscopy. Scraping of the cells was done in 10 ml of PBS after their washing with PBS, and the same procedure was followed as for the trypsinized cells.

RESULTS

Changes in Phosphate Bands. Study of the frequency region 1000–1350 cm^{-1} revealed significant differences in the infrared spectra between normal and malignant colonic tissue. Fig. 1, showing the superimposed infrared spectra of a pair of normal and malignant tissue samples from one patient, illustrates these differences. The strongest bands are at 1241.0 cm^{-1} and 1082.4 cm^{-1} (frequencies in normal tissue). They are due to the asymmetric ($\nu_{as}PO_2^-$) and symmetric ($\nu_sPO_2^-$) phosphate stretching modes, respectively (14). In all cases, when compared with normal tissues, the malignant tissues displayed decreased intensity of the $\nu_{as}PO_2^-$ band, increased intensity of the $\nu_sPO_2^-$ band, and changes in the shape of these bands, as shown in Fig. 1. In addition, the frequencies of these bands were shifted in malignant tissues. The $\nu_{as}PO_2^-$ band was shifted from 1241.0 cm^{-1} in the normal tissue to 1239.4 cm^{-1}, while the $\nu_sPO_2^-$ band was shifted from 1082.4 cm^{-1} to 1085.1 cm^{-1}.

The intensity of these peaks varied from patient to patient and also within the same tumor sample. The ratio of the peak intensity of these two bands varied as well. The variation in the intensity and band shape in this frequency region may reflect the variation in the proportion of nonmalignant and nonepithelial cells present in each tissue section.

These bands originate mainly in the phosphodiester backbone of cellular nucleic acids (14). The contribution of these bands by phosphate residues present in membrane lipids is negligible, as indicated by the relative intensities of other vibrational modes of membrane lipids. For example, the peak intensity ratio between the $\nu C{=}O$ band and the $\nu_{as}PO_2^-$ band of phospholipids is 1.5–1.8. In the spectra of human colon tissues, this ratio is 0.13–0.34. This finding indicates that phospholipids do not contribute appreciably to the intensity of the $\nu_{as}PO_2^-$ band in the infrared spectra of human colon tissues. Since the intensity of the $\nu_sPO_2^-$ band is slightly

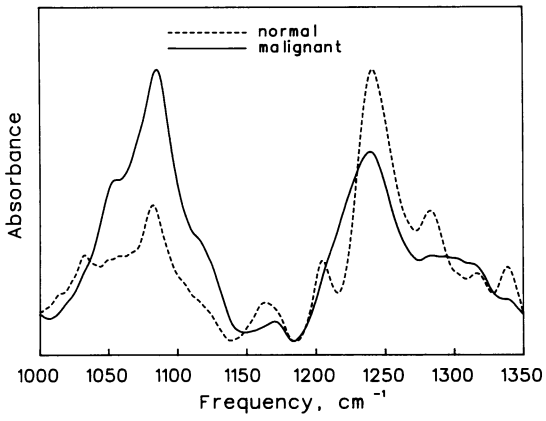

FIG. 1. Infrared spectra of tissue sections from colon adenocarcinoma and histologically normal mucosa 10 cm away from the tumor (frequency region, 1000–1350 cm^{-1}; auto-scale plotting configuration, in which peak intensities are automatically normalized to the highest peak).

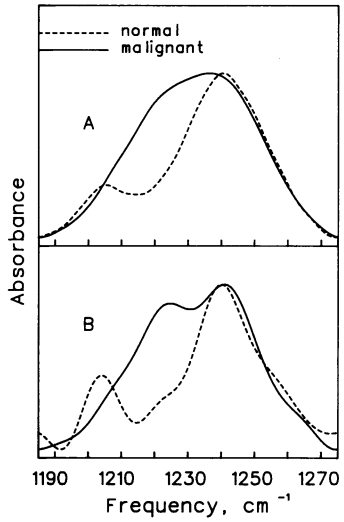

FIG. 2. Infrared spectra of a pair of normal and malignant colon tissues (frequency region, 1185–1275 cm^{-1}; auto-scale plotting). (*A*) Original spectra. (*B*) Spectra after band narrowing with Fourier self-deconvolution as described in text.

weaker than that of $\nu_{as}PO_2^-$ in phospholipids, phospholipids also do not contribute to the intensity of the $\nu_sPO_2^-$ band in the infrared spectra of human colon tissues.

The enlarged spectra of the $\nu_{as}PO_2^-$ band of a pair of normal and malignant colonic tissues are plotted together in Fig. 2. Fig. 2*A* shows the original spectra, whereas Fig. 2*B* shows the corresponding $\nu_{as}PO_2^-$ spectra after band narrowing using Fourier self-deconvolution with an enhancement factor of 1.45 and band width of 20 cm^{-1} (15). In malignant tissue this band splits into two at 1241.1 and 1223.8 cm^{-1}; such splitting is barely discernible in normal tissue. The frequency of the $\nu_{as}PO_2^-$ mode is at 1240–1260 cm^{-1} when it is completely non-hydrogen-bonded and at about 1220 cm^{-1} when it is fully hydrogen-bonded (16). Therefore, in these malignant colonic tissues, the hydrogen-bonding of the oxygen atoms of the phosphate backbone of nucleic acids is increased as indicated by the increase in the intensity of the 1223.8 cm^{-1} band. This is in contrast to the situation in the corresponding normal colonic tissue.

Fig. 3 shows the pressure dependences of the frequencies of the two $\nu_{as}PO_2^-$ component bands of malignant colonic tissue. The frequencies of these bands were calculated from the third-power derivative spectra with a breakpoint of 0.3 (15). The frequency of the band at 1223.8 cm^{-1} decreases with increasing pressure. This provides further evidence that this

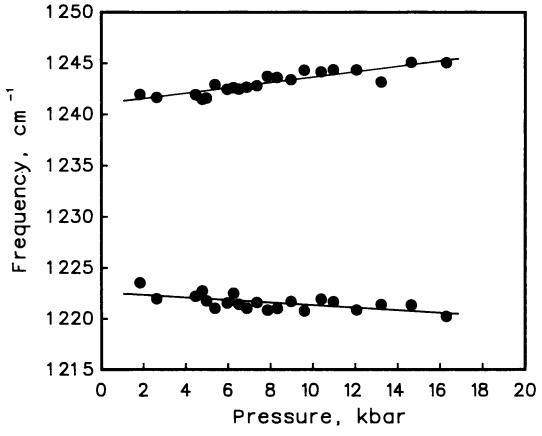

FIG. 3. Pressure dependences of the asymmetric phosphate stretching frequencies of malignant colon tissue.

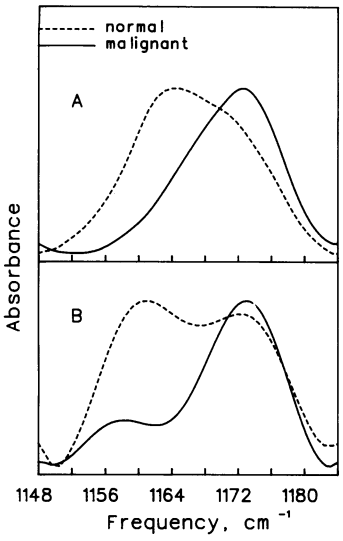

FIG. 4. Infrared spectra of a pair of normal and malignant colon tissues (frequency region, 1148–1184 cm^{-1}; auto-scale plotting). (*A*) Original spectra. (*B*) Spectra after band narrowing with Fourier self-deconvolution as described in text.

band is due to the $\nu_{as}PO_2^-$ mode of the hydrogen-bonded phosphate backbone of nucleic acids (17). The frequency of the 1241.1 cm^{-1} component band increases with increasing pressure, which is the result of compression of the chemical bonds in non-hydrogen-bonded PO_2^- groups (17, 18).

Changes in the C—O Band. The relatively weak band at 1164.2 cm^{-1} (this is its frequency in normal tissue) is due to the stretching mode (νC—O) of C—O groups present in cell proteins (14). The νC—O band of the acyl chains of membrane lipids appears in this frequency region as well. However, the contribution to this band by membrane lipids is negligible, as indicated by the relative intensities of the νC—O and νC=O modes of membrane lipids. The peak intensity ratio between the νC=O band and the νC—O band of lipids is in the range 5.2–12.4, whereas in the spectra of human colon tissues this ratio is 0.6–0.8. Consequently, the νC—O band in the spectra of human colon tissues is essentially from the νC—O mode of cell proteins. In malignant tissue this band changes in both shape and peak maximum. The peak maximum shifts from 1164.2 cm^{-1} in normal tissues to 1173.1 cm^{-1} in malignant tissues. Fig. 4 shows the enlarged spectra of this band. Fig. 4*A* shows the original spectra, whereas Fig. 4*B* shows the corresponding spectra after band narrowing using Fourier self-deconvolution with an enhancement factor of 1.4 and a band width of 20 cm^{-1} (15).

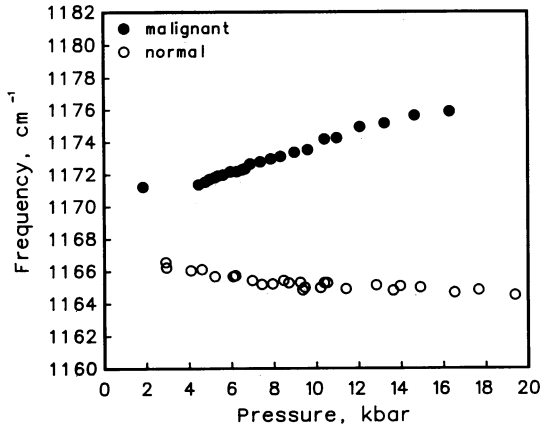

FIG. 5. Pressure dependences of the center of gravity of the νC—O bands of a pair of normal and malignant colon tissues.

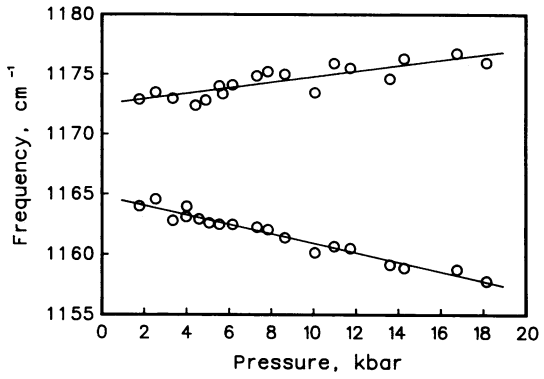

FIG. 6. Pressure dependences of the frequencies of the νC—O component bands of normal colon tissue.

Study of the pressure dependence of spectral parameters of these tissues reveals changes in both the position of the center of gravity and the peak maximum of these bands. When pressure is applied to the tissue, the center of gravity of this band moves towards lower frequencies in normal tissue. In contrast, in malignant tissues the center of gravity shifts towards higher frequencies (Fig. 5). The frequencies in Fig. 5 were calculated from the third-power derivative spectra with a breakpoint of 0.2 (15).

The deconvoluted spectra (Fig. 4*B*) show that the νC—O band consists of two overlapping bands in both normal and malignant tissues. The intensity of the low-frequency component band is dramatically decreased in malignant tissues. The pressure dependence of the frequencies of these two component bands of normal colonic tissue, calculated from the third-power derivative spectra with a breakpoint of 0.3 (15), is shown in Fig. 6. The frequency of the high-frequency component band increases with increasing pressure, while that of the low-frequency component band decreases with increasing pressure. In general, the vibrational frequency of a C—O functional group in a molecule is increased by external pressure because of the compression of the chemical bond of the functional group and the pressure-enhanced intermolecular interactions. On the other hand, if the functional group is hydrogen-bonded, its vibrational frequency is decreased. With increasing pressure, the vibrational frequency of the functional group is decreased further (17). The results shown in Figs. 4 and 6 indicate that the low-frequency component band is due to the νC—O mode of the hydrogen-bonded C—O groups, whereas the high-frequency band is due to the νC—O mode of the non-hydrogen-bonded C—O

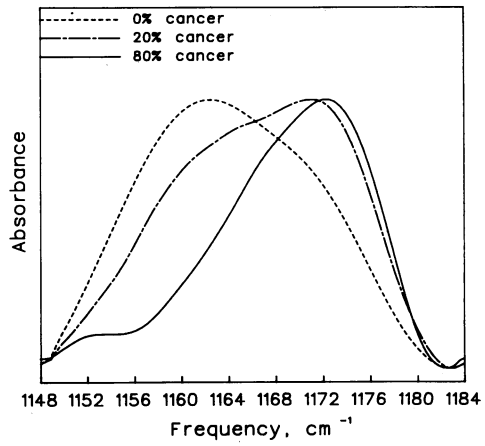

FIG. 7. Infrared spectra of the νC—O band of three colonic tissue samples differing in the proportions of normal and malignant components. These samples are from the same patient.

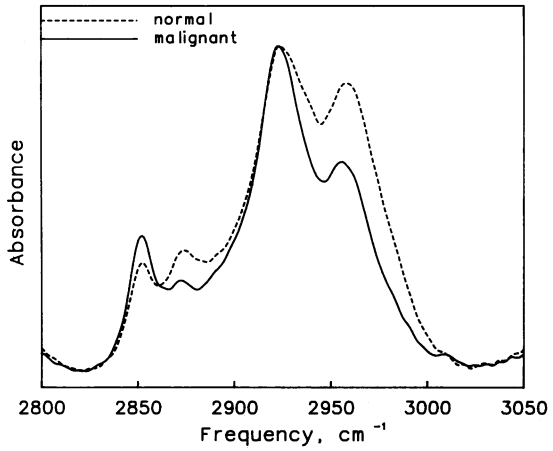

FIG. 8. Infrared spectra of a pair of normal and malignant colon tissues (frequency region, 2800–3050 cm^{-1}; auto-scale plotting).

groups. The dramatic decrease in the intensity of the low-frequency component band in malignant tissues suggests that in colon cancer most of the hydrogen bonds in the C—O groups have disappeared.

The integrated intensity of the lower frequency component band decreases as the proportion of malignant cells in a tissue sample increases (Fig. 7).

Changes in the CH Stretch Region. Fig. 8 shows the infrared spectra of a pair of normal and malignant colonic tissues in the CH stretching region. The band at 2852.5 cm^{-1} is due to the symmetric CH_2 stretching mode ($\nu_s CH_2$) of the methylene chains in membrane lipids. The band at 2958.5 cm^{-1} is due to the asymmetric stretching mode of the methyl groups ($\nu_{as} CH_3$). The $\nu_{as} CH_3$ modes of both the end-methyl groups of membrane lipids and the methyl groups of proteins are at about the same frequency (14, 17). Therefore, the $\nu_{as} CH_3$ mode of methyl groups of both membrane lipids and proteins contribute to the intensity of the band at 2958.5 cm^{-1}.

In malignant colonic tissues, the intensity of the $\nu_{as} CH_3$ band is decreased while that of the $\nu_s CH_2$ band is increased, when compared with the corresponding bands in normal tissue (Fig. 8). This indicates that the ratio of the number of methyl groups to that of methylene groups is decreased in malignant tissue, compared with normal colonic tissue. While this ratio is decreased in the malignant tissue of all pairs of normal and malignant tissues that we studied, its absolute value varies from patient to patient.

Pressure Dependence of the CH_2 Bending Mode. The pressure dependence of this mode has been widely used to

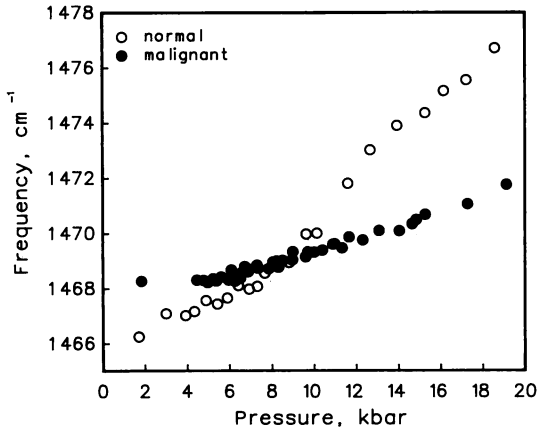

FIG. 9. Pressure dependences of the CH bending frequencies of methylene chains of membrane lipids of a pair of normal and malignant colon tissues.

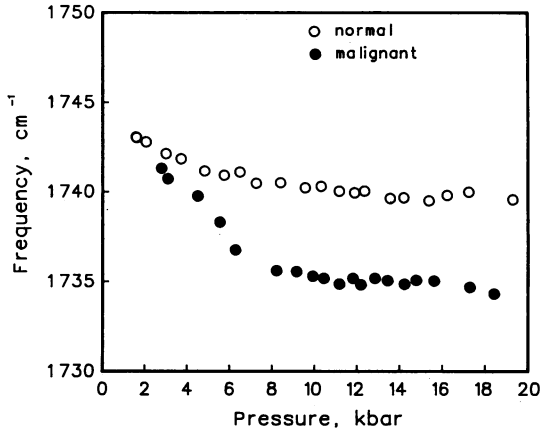

FIG. 10. Pressure dependences of the C=O stretching frequencies of methylene chains of membrane lipids of a pair of normal and malignant colon tissues.

investigate the packing characteristics of methylene chains in membrane lipids (19, 20). Fig. 9 shows the pressure dependence of the CH_2 bending mode of the methylene chains in membrane lipids in a pair of normal and malignant colonic tissue. The frequencies were obtained from the third-power derivative spectra with a break point of 0.4 (15). At atmospheric pressure the frequency of this band is higher in the malignant tissue than in its normal counterpart; this relationship is reversed as pressure increases. The range of relative change of the frequency of this mode over a wide range of pressures is much more restricted in the malignant tissues compared with the normal controls (in this example, in the normal tissue the increase in frequency of this mode in going from atmospheric pressure to 20 kbar is 4 times greater than that noted in malignant tissue). These findings indicate that in malignant tissues at atmospheric pressure, the conformational structure of the methylene chains of membrane lipids is more disordered than in normal tissues. As pressure increases the interchain interactions are enhanced as shown by the increase of this frequency. The pressure-enhanced interchain interactions is larger in the more conformationally ordered normal tissue (19, 20).

Pressure Dependence of the C=O Stretching Mode. These frequencies were obtained from the third-power derivative spectra with a breakpoint of 0.3. The intensity of the C=O stretching mode (νC=O) in the spectra of tissues arises from the vibration of the C=O groups of the acyl chains in membrane lipids (17, 18). Sometimes, however, the νC=O mode of tissue triglycerides presenting as microscopic oil drops, contributes to the intensity of this band. The data of the νC=O band shown in Fig. 10 were obtained from a pair

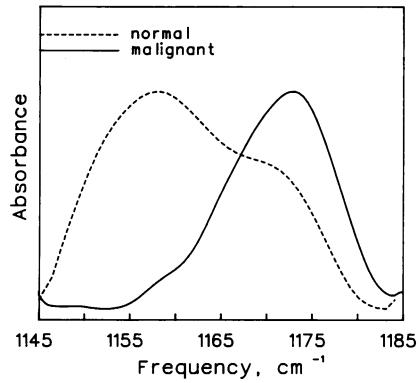

FIG. 11. Infrared spectra of the νC—O bands of cultured colon fibroblasts and the LoVo colon adenocarcinoma cell line.

of normal and malignant tissues from a single patient after the oil droplets were removed by carefully washing the tissue samples with ^2H_2O. The intensity of this band varied from patient to patient. In both normal and malignant tissues, the frequency of this band decreased with increasing pressure; however, this decrease was more pronounced in malignant tissues. The latter finding indicates that the hydrogen-bonding of the acyl C=O groups is stronger in malignant tissues compared with normal tissues (17, 18).

IR Spectra of Colon Cell Lines. To further evaluate these findings, an infrared spectroscopic study of a series of cultured human colon cell lines was undertaken. Details will be given elsewhere. Many of the infrared spectroscopic features are common to both the cultured cell lines and the colonic tissues that we studied. For instance, the νC—O bands of cultured colon fibroblasts and the LoVo cell line are compared in Fig. 11. These spectra are similar to those of colonic tissue shown in Fig. 4*A*.

DISCUSSION

Our data demonstrate that colorectal cancers display abnormal FT-IR spectra compared with normal control tissue from the same individual. The entire spectrum of the evolution of colon cancer—i.e., the various forms of adenoma—have not been examined yet to know whether they also manifest these spectral abnormalities.

The spectral changes that we have observed reflect alterations in the structure of important informational and structural molecules in the malignant tissue. They involve the degree of hydrogen-bonding of (*i*) oxygen atoms of the backbone of nucleic acids (they are the two oxygen atoms bound to P that do not participate in the phosphodiester bond); (*ii*) C—O groups in cell proteins; and (*iii*) the C=O groups of the acyl chains of membrane lipids. There are also additional changes in the structure of proteins and membrane lipids, as judged by the changes in their ratio of methyl to methylene groups. Finally, the packing and the conformational structure of the methylene chains of membrane lipids are changed in colonic malignant tissue. Although our findings do not allow a more specific assignment of these changes, nevertheless they indicate extensive chemical and physical changes in the malignant colonic tissue, and, in a way, attest to the complexity of the malignant phenotype. It is interesting to note that the severe reduction in hydrogen bonding of C—O groups of proteins in malignant colon tissue can involve only three amino acids—namely, tyrosine, serine, and threonine. The OH groups of these three amino acids, which form the C—O bonds monitored here, are phosphorylated by several oncoproteins, an event considered important in carcinogenesis (21).

We do not know the cell type responsible for the changes in the FT-IR spectra of malignant colonic tissues. There are two reasons to suspect that these changes may originate in the malignant colonocyte *per se*: (*i*) the integrated intensity of the C—O band in malignant tissue changes in tandem with the proportion of malignant cells present in a given tissue section, and (*ii*) the spectral characteristics of cultured colon cancer cell lines are, in general, similar to those observed in tissue sections. Regarding the latter point, however, it should be noted that, besides the general problem of extrapolating tissue culture findings into intact tissues, our colon cancer cell lines were compared with normal colonic fibroblasts (for lack of a reliable culture colonic epithelial cell). Ongoing work is attempting to assess directly the cellular origin of the observed spectral abnormalities.

Whatever the exact nature of the physical and chemical alterations in the malignant colonic tissues, our study has two practical implications. First, it demonstrates that it is feasible to apply productively pressure-tuning FT-IR spectroscopy to the study of human tissues. This approach provides the opportunity to monitor simultaneously several chemical and physical parameters of a tissue with very small samples. Second, it suggests that determination of these spectra may be of some use in rapid evaluation of the malignant phenotype in colonic tissue and perhaps other tissues.

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