Cancer field effects in normal tissues revealed by Raman spectroscopy

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Abstract: It has been demonstrated that the presence of cancer results in detectable changes to uninvolved tissues, collectively termed cancer field effects (CFE). In this study, we directly assessed the ability of Raman microspectroscopy to detect CFE via in-vitro study of organotypic tissue rafts approximating human skin. Raman spectra were measured from both epidermis and dermis after transfer of the rafts to dishes containing adherent cultures of either normal human fibroblasts or fibrosarcoma (HT1080) cells. Principal components analyses allowed discrimination between the groups with 86% classification accuracy in the epidermis and 94% in the dermis. These results encourage further study to evaluate the Raman capacity for detecting CFE as a possible tool for noninvasive screening for tumor presence.

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1. Introduction

The idea that histologically normal tissue is biochemically altered by signals from adjacent tumor cells was hypothesized roughly 50 years ago [1]. Since that time, a number of reports have validated the existence of several signaling mechanisms and pathways involved in response to cancer [2]. These responses can be comprised by the collective phrases malignancy-associated changes (MAC) or cancer field effects (CFE): the concept that some of the biological variables involved in neoplastic lesion formation have an effect on nearby tissues. This phenomenon could potentially enable screening and diagnosis of cancer via interrogation of readily accessible tissues, such as skin. The majority of CFE studies have relied on tissue processing or cellular isolation, however, neither of which fully account for the intrinsic behaviors of intact tissue nor can be easily translated to clinical application. While CFE present a significant potential for novel cancer diagnostics, few studies to-date have demonstrated CFE detection methods that could be used in intact tissue.

One method that has been clinically demonstrated for its ability to detect CFE in tissues is elastic light scattering. Backman et al. have performed considerable work using a variant of this phenomenon to show that tissues near a tumor are different from those further away from the tumor [3–5]. Because the method employed is keenly sensitive to changes in particle size within the field-of-view, they attribute the optical changes to microvasculature associated with tumorigenesis. Based on these detectable changes, they have demonstrated diagnostic capabilities in healthy tissues as far as 30 cm away from the primary tumor [3], as well as the ability to detect cancer in an animal model well before it could be realized using conventional methods [4,5]. Yet elastic scattering is based on the morphology of the cellular and tissue environments, features that change in response to biochemical initiation. By utilizing an optical technique that is capable of directly measuring the biochemistry within the cells and tissues, it may be possible to increase the sensitivity to CFE, both spatially and temporally.

In recent years, there has been increasing interest in the use of Raman spectroscopy for tissue diagnostics. These studies have shown that features of the Raman spectrum can be related to molecular and structural changes associated with neoplastic transformation. Recent reports have demonstrated the successful application of Raman spectroscopy for disease characterization in vivo in vasculature [6], cervix [7], breast [8], and skin [9], among others. More interesting, however, is that the Raman spectra of histologically normal tissues surrounding tumor have been shown to differ from the Raman spectra of tissues more distal to the tumor [10].

Combining the Backman group’s success using optical methods that probe particle size and morphology to detect CFE, the fact that these physical changes would be preceded by molecular changes, and the molecular specificity of Raman spectroscopy, it has been hypothesized that these findings are supportive of Raman detection of CFE. In this work, we explore the effects of cancer presence on the Raman spectra of engineered tissue rafts. By culturing these organotypic rafts in proximity to-, but physically separated from cancerous...
cells, we are able to minimize extrinsic variables and directly assess the ability of Raman spectroscopy to detect CFE.

2. Experimental

The organotypic tissue culture raft models (“rafts”) used in this study were created according to published protocols [11]. In brief, these rafts consisted of a stromal equivalent containing a collagen-matrix with embedded fibroblasts, over which an epidermis of differentiated keratinocytes was grown. The rafts were incubated on a wire mesh suspended approximately 3 mm above the bottom of a 60 mm culture dish. Culture media was added (~8 mL) such that it reached the wire mesh but no part of the raft was submerged. Rafts were differentiated under normal conditions for 8 days (as per protocol), after which the mesh grids containing the rafts were transferred to 60 mm dishes containing adherent cultures of either normal fibroblasts (derived from human skin) or fibrosarcoma (HT1080) cells. At the time of transfer, both the fibroblast and HT1080 cultures were ~70% confluent. Media was added, as before, only to the level of the mesh. For clarity, those rafts transferred to dishes containing fibroblasts are termed “naïve” throughout this manuscript, while those co-cultured with the HT1080 cells are termed “tumor-associated.” To ensure isolation between the rafts and the plated cells, the rafts were transferred to new dishes with adherent cultures of the same respective cell types (~70% confluence) at each change of media, every 2 days. Adhesion of the cells to the dish was confirmed by inverted phase microscopy of each dish prior to any spectral measurements, thus ensuring that the cultured cells did not infiltrate the rafts.

Rafts were created in 6 separate dishes, equally divided between naïve and tumor-associated, with 2 rafts per dish. The rafts were created such that the epidermis did not completely overlap the dermis, thereby allowing spectral measurement of the two strata without need for a confocal setup and increased measurement times. Three spectral measurements were obtained from both dermis and epidermis of each raft, at multiple positions around the raft. Thus, each population (naïve, tumor-associated) yielded 18 epidermal spectra and 18 dermal spectra on each day of measurement. The spectral measurements were performed before the rafts were associated with the plated cells, and every 1-3 days until analysis of that day’s raft spectra revealed a statistical change between the naïve and tumor-associated populations, after which no subsequent spectra were obtained. Spectra were also measured of the isolated cells used in the adherent cultures (normal fibroblasts and HT-1080). These cells were smeared onto a fused quartz slide and five measurements of each cell type were obtained from various positions around the smear.

Raman measurements were obtained using a Raman microspectrometer (RM1000, Renishaw PLC, Wotton-under-Edge, UK). In brief, this system is based on an upright microscope frame with attached spectrometer, and a 785 nm laser providing approximately 40 mW illumination power to the sample, through a 20 × , 0.4 NA objective (model 566026, Leica Microsystems, Wetlzar, Germany); the system was operated in non-confocal mode. Spectral measurements were obtained using 30-60 sec. integration, from 600 to 1800 cm\(^{-1}\), binned to 3 cm\(^{-1}\). For each experiment, the instrument was calibrated for spectral dispersion (Ne:Ar lamp), Raman shift (acetaminophen), and spectral response (NIST 2241 glass). Instrument background signal was subtracted from each tissue spectrum. Fluorescence background was subtracted via the modified polynomial fitting method [12], using the full wavenumber range and 5th order fit. High order (shot) noise was removed using a 2nd order Savitzky-Golay smoothing filter. All processed spectra were normalized to respective mean intensity and mean centered prior to statistical comparison.

The spectral data set was analyzed each measurement day using principal components analysis (PCA) in the Matlab (Mathworks Inc., Natick, MA) programming environment (the epidermal and dermal measurements were evaluated separately). That day’s full spectral data set was input with each wavenumber intensity representing a separate predictor variable, using leave-one-out cross-validation. PCA scores were used to assess diagnostic accuracy of the
Raman spectra, via definition of decision lines that maximized separation of the naïve and tumor-associated raft populations with a minimum of classification error. Sensitivity and specificity were calculated using this class separator. Because there were only two classes evaluated, it is important to note that these sensitivity and specificity values are interchangeable. Overall classification accuracy was also calculated as the total number of correctly classified measurements per total measurements each day.

3. Results

Statistical analysis of the Raman spectra produced no significant discrimination between the populations’ epidermal or dermal measurements until 8 days after co-culture with the respective plated cells. Figure 1(A) shows the mean Raman spectra of both epidermal populations after 8 days of co-culture. Qualitatively, there are slight spectral differences between the two populations that are more readily visualized in Fig. 1(C), which shows the mean difference spectrum (tumor-associated minus naïve). The most apparent difference is seen in amino acid bands near 855 cm$^{-1}$ (tyrosine, proline) and 1004 cm$^{-1}$ (phenylalanine). The 1004 cm$^{-1}$ band has also been attributed to the C–C vibration of keratin [13]. There is also some change in the amide III, protein, and lipid band region between approximately 1250 and 1340 cm$^{-1}$.

Figure 1(B) shows the mean Raman spectra measured on the dermis that protruded slightly from the perimeter of the epidermis. These spectra are qualitatively more dissimilar between the naïve and tumor-associated populations than in the epidermis, the regions of which are more readily seen in the difference spectrum in Fig. 1(D). The dermal difference spectrum identifies many of the same general Raman bands shown in the epidermis, with slight variation. The dermal spectra are most different in the 858 cm$^{-1}$ shoulder of the 855 cm$^{-1}$ tyrosine/proline peak, and in the 1018 cm$^{-1}$ neck region between the 1004 cm$^{-1}$ phenylalanine/keratin peak and the 1040 cm$^{-1}$ peak attributed to both proline [14] and...
oxidized albumin [15]. In addition to the general differences in the broad amide III region, the dermal spectra also show more distinct disparity in the CH\textsubscript{2} and CH\textsubscript{3} deformations revealed in the 1420 cm\textsuperscript{-1} region.

Plotting of the principal component (PC) scores reveals a clear separation of the two raft populations in both the epidermal and dermal spectra. As seen in Fig. 2(A), a decision line separating the epidermal measurements of the two populations accurately discriminates 17/18 tumor-associated raft spectra, and 14/18 naïve spectra, for a sensitivity and specificity of 94% and 78%, respectively, and an overall classification accuracy of 86% (31/36). Figure 2(B) shows the application of a decision line to the PC scores of the dermal measurements, which yield a higher discriminant accuracy of 18/18 tumor-associated raft spectra and 16/18 naïve raft spectra, for a sensitivity and specificity of 100% and 89%, respectively, and an overall classification accuracy of 94% (34/36).

The loading plots of the PCA, seen in Fig. 2(C) (epidermis) and 2D (dermis) reveal that the Raman band regions responsible for the PC score discrimination are largely the same as those regions identified in the difference spectra. In addition, the epidermis PCs load on the
phospholipid and nucleotide band near 698 cm$^{-1}$, the 936 cm$^{-1}$ peak of proline, valine, and glycogen, the 1095 cm$^{-1}$ peak of the DNA phospholipids backbone, and the CH$_2$ bending mode of proteins and lipids near 1448 cm$^{-1}$. The dermal spectral loadings are nearly identical to the difference spectra, adding the strong amide I band near 1660 cm$^{-1}$.

The mean Raman spectra of both the normal fibroblasts and the HT1080 fibrosarcoma cells are shown in Fig. 3(A). The difference spectrum of these cells, shown in Fig. 3(B), shows much higher relative intensities and dissimilar band locations as compared to the mean difference spectra from the raft measurements. PCA of the cellular spectra allowed complete discrimination between the cell lines using the first two PCs. The peak locations exhibiting high loadings are marked on the spectra. These include the nucleotide peak at 783 cm$^{-1}$, the tyrosine peaks at 827 and 851 cm$^{-1}$ (also representing DNA backbone and proline), the wide shoulder band of the protein amide III band near 1257 cm$^{-1}$, and the CH$_2$ peak of lipids and proteins centered near 1445 cm$^{-1}$. Applying this PCA model to the raft spectra yielded no discriminant ability (~50% error) for either the epidermal or dermal measurements, suggesting that the cells themselves were not responsible for the discrimination between the raft populations.

4. Discussion

Optical methods such as Raman spectroscopy have an ever-increasing body of support in scientific literature for their abilities to successfully discriminate tissue pathology. While the noninvasive nature and capacity for automated diagnosis based on biochemistry is the hallmark advantage of optical techniques, there remains the physical mandate that light must be transmitted to- and received from the tissue of interest. Thus the majority of reports on so-called “optical biopsy” target readily accessible organs and tissues such as skin, gastrointestinal and gynecologic tracts. But for those other tissues, more technical complexity in light delivery/collection is necessary, and oftentimes requires at least some minimal invasion. Thus, the motivation behind this work: if a tumor causes changes to surrounding
tissues that can be detected by the Raman technique, then presumably that tumor could be detected by performing optical biopsy of an accessible tissue region. Such capabilities have been indirectly indicated in previous clinical Raman studies, including those of the author and former colleagues [9,10]. In this report, we confirm the Raman capacity to detect changes in normal tissues caused by adjacent tumor.

The raft model was chosen for this study to maximize control over unassociated variables. The clinical Raman studies included in the paper by Keller et al. provide circumstantial evidence of Raman detection of CFE, but the referenced studies were not controlled for this purpose and, as such, could not accurately posit the correlation between Raman spectral changes and CFE. This same work also includes a comparison of Raman spectra obtained from raft cultures created with and without cancerous cells in the epidermis and dermis. While no discrimination was performed, their findings that the spectral changes are more intense in the dermis than in the epidermis mirror the findings presented here.

While the experimental model used in this study does allow validation of the effect of cancerous cells on non-cancerous tissues, the reliance on medium as a conductor between the adherent cell monolayers and the tissue rafts is not ideal for understanding progression of CFE in-vivo. Particularly, the volume of the medium used in the model and its rate of exchange may effect the time at which the spectral changes necessary for discrimination could arise. Furthermore, the use of medium as conductor minimizes the characterization of cell-to-cell transmittance of CFE, as would occur in solid tumors and surrounding tissues. It is important to note that these abovementioned reasons minimize the temporal import of our findings, in that slight changes of our model may increase or decrease the number of days at which the Raman spectra can reliably register a change. Regardless of the model’s shortcomings, however, it does allow proof-of-concept of the Raman detection of CFE.

In order to avoid any chance raft-to-raft spectral differences from obscuring our intended experimental result, we took multiple steps in the experiment design and analysis. First, we separated the rafts into multiple dishes, thus any unforeseen changes in one dish would not contaminate the entire population spectral data set. Second, we placed two rafts in each dish, thus any anomalies in one raft could be verified against another within the same dish. Third, and perhaps most important, we attempted to discriminate the raft populations’ spectra not only after co-culture with the plated cells but also before the rafts were co-cultured with the plated cells, and could not produce a significant discriminant result until the stated 8 days of co-culture, even using less conservative statistical tools such as partial least squares discriminant analysis (PLSDA) or linear discriminant analysis (LDA) of the PCA scores. Based on these safeguards, we can reliably assert that the changes seen between the two populations are the result of association with cancerous cells.

The spectral changes that resulted in discrimination between the two populations did not present gradually, as might be expected. Rather, the spectra did not produce any significant discrimination (e.g. classification error ~50%) over the first seven days after co-culture with the plated cells, then showed a clear distinction on the 8th day. This is most likely due to detection limits of the Raman technique, whereby the slight changes over the first 7 days did not produce the magnitude of biochemical change necessary to overcome inherent noise of the system and sample. Similarly, measurement of the media from the raft dishes produced relatively low Raman signal, and the spectra did not allow discrimination between the populations. Continuing experiments in the raft models and in animal or human studies will evaluate the progression of these changes temporally and spatially, to further elucidate CFE distribution.

Many of the spectral features that are identified as diagnostically relevant are similar to those identified in many Raman studies of various pathological tissues, such as the amide bands, prominent CH$_2$ peak, amino acids, and bases and backbone of nucleic acids. This is obvious given that these elements largely form the basis of biological materials. Yet the Raman band near 1040 cm$^{-1}$ that presents in the dermal measurements is not typically
identified in previous tissue studies. In many cases, the region of approximately 1030 to 1070 cm$^{-1}$ is attributed to fused silica from the instrument optics, but because this study accounted for background signal it is unlikely to be the source of the band. Frank et al. found this feature in ex vivo measurements of breast tissue and attributed it to proline [14]. However, their samples were fixed in formalin prior to measurement, which has previously been shown to produce a tissue peak near 1040 cm$^{-1}$ related to the formalin [16]. An in vivo skin Raman study has identified this peak to be a result of albumin oxidation, and tested this attribution by measuring purified cysteic acid, known to cause delinking of the albumin disulfide bonds [15]. Nearby peaks also appear in published spectra of cervix, where a band at 1048 cm$^{-1}$ is attributed to glycogen [10], and in skin, where a peak at 1032 cm$^{-1}$ is identified as C–C stretching modes of keratin [13]. It remains to be determined whether this Raman spectral feature is related to the CFE, or whether it is an artifact in cultured tissues due to the fortification of cell and tissue culture media with bovine albumin. This will most likely be answered as these studies progress from the Petri dish into animal or clinical analyses.

Based on the changes described previously using various raft models [10,11] as well as the results of this study, the Raman spectral changes can be attributed to CFE with more certainty. Further study is obviously warranted to broaden characterization of these changes, including their reversibility, the manner of their spread within and beyond tissues, the distance at which these changes can be detected, and whether the CFE are unique to the tumor type. Beyond this basic exploration, study in animal and/or clinical studies can further elucidate the nature of CFE detectability in multi-tissue and trans-organ environs, including the effects of hemodynamics and lymphatic circulation. Exploitation of this effect could potentially allow the use of Raman spectroscopy for rapid screening and diagnosis of internal tumors by measuring readily accessible anatomy.

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