Continuous monitoring of WBC (biochemistry) in an adult leukemia patient using advanced FTIR-spectroscopy

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Abstract

Fourier transform infrared (FTIR)-spectroscopy has been found useful for monitoring the effectiveness of drugs during chemotherapy in leukemia patients. In the present work, spectral changes that occurred in the white blood cells (WBC) of an adult acute myeloid leukemia (AML) patient and their possible utilization for monitoring biochemistry of WBC were investigated. The phosphate absorbance from nucleic acids and the lipid–protein ratio in the WBC decreased immediately after treatment and then increased to levels of a control group. Similar observations were recorded in child patients with acute lymphoblastic leukemia (ALL) who were used as test cases. These parameters maybe used as possible markers to indicate successful remission and suggest that FTIR-spectroscopy may provide a rapid optical method for continuous monitoring or evaluation of a WBC population.

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1. Introduction

Leukemia is characterized by a large number of immature cells (blasts) in the blood. The cells are in a different growth stage compared to what are normally present in the blood. The changes in tissue biochemistry are reflected as spectral changes. This is the principle behind several diagnostic tools that are based on spectroscopic methods. Fourier transform infrared (FTIR)-spectroscopy is one such area that has seen rapid development in the past decade with a promise of easier, rapid and objective diagnosis [1]. FTIR-spectroscopy is also an effective and non-destructive method to monitor cellular changes [2,3]. Diseases of several organs have been identified using structure and quantity of biomolecules in biological samples such as proteins [4], nucleic acids [5] and lipids [6].

Chemotherapy decreases the blast count [7,8]. Chemotherapeutic drugs such as doxorubicin and vincristine interfere with cell division by binding to the DNA and targeting its synthesis or function [7]. In non-dividing cells; however, the lethal effects are due to their ability to interfere with DNA repair polymerases as well as lipid biosynthetic enzymes. Thus, the presence of blasts in the blood is used as an indicator of residual malignancy during treatment and care of leukemia patients after induction of chemotherapy. However, no studies have been carried out to examine the biochemistry of white blood cells (WBC) to see whether they become biochemically normal following chemotherapy, though conventional blood profiles are studied. Rapid and continuous monitoring of biochemistry of WBCs on a molecular level remains a challenge, which is also essential for the well being of the patient. Thus, we studied FTIR-spectroscopy as a potential reagent...
free supplementary technique for such routine monitoring of WBCs in patients being treated for leukemia.

FTIR-spectroscopy along with cluster analysis of the spectra has shown a promise as a tool for monitoring the effectiveness of drugs during chemotherapy in children [9,10]. However, no such report on the validation of the technique for monitoring biochemical changes in WBC during chemotherapy of adults is available. It is well known that the response of adults to leukemia is different from children and often more difficult. In the present work, we study the utility of FTIR-spectroscopy derived parameters for an adult, which were earlier found effective in monitoring changes in WBCs during chemotherapy in children. The patient in the present case study had low blast count in the peripheral blood (5%), which is only a minor deviation from normal values, thus, this patient might serve as an analogy for the effects of chemotherapy on WBCs in vivo in normal adults. The plasma and RBCs profiles before the treatment regimen were comparable to the normal average value with low WBC counts. For further validation of the FTIR-spectroscopy derived markers a comparison is made with the values obtained from a control population. The corresponding values of the diagnostic parameters from healthy persons are obtained in parallel to act as control references to ascertain the presence of residual malignancy or the return to normalcy. Thus, it becomes imperative that monitoring by simple blood count would not be possible, and an optical method for following the biochemical changes in WBCs would be more suitable. To further substantiate this hypothesis, cases of child leukemia patients under treatment were tested.

2. Materials and methods

The blood samples were collected from the adult patient with his consent. The adult was given a treatment regimen the “7 and 3” protocol composed of Arabinoside-C (Ara-C) for the first 7 days and Daunorubicin (Dauno) for the first 3 days as shown in Scheme 1. Blood from a group of normal people (eight healthy adults without any known clinical symptoms for any disease) was taken to serve as a control for comparison. Blood samples from two children under treatment for acute lymphoblastic leukemia (ALL) were taken after obtaining consent of their parents and used as test cases. Erythrocytes, plasma and WBCs from blood of leukemia patients and controls were separated using the method of Hudson and Poplack [11] within two hours of the collection of the blood samples. The WBCs were washed with normal saline (0.9 M sodium chloride) to remove any impurities from the plasma. The blood components were then diluted as required in normal saline. Two microlitres of each blood component was spotted on a zinc selenium slide to form approximately a monolayer of cells and air dried under the laminar flow to remove remaining water.

The FTIR measurements on samples were performed using the FTIR microscope IRscope II with liquid nitrogen cooled mercury-cadmium-telluride (MCT) detector, coupled to the FTIR spectrometer (Bruker Equinox model 55/S, OPUS software). To achieve high signal to noise ratio (SNR) 128 co-added scans were collected in each measurement in the wavenumber region 600–4000 cm⁻¹ for Fourier transform processing. Five sites were measured on each sample containing an average 100 cells as reported previously with an aperture of 1.5 mm that measures a circular area of 100 microns diameter [9,10]. The spectra were baseline corrected using OPUS software and were normalized to the amide II (~1545 cm⁻¹) absorbance band in the region 800–1800 cm⁻¹. The averages of the normalized spectra were used for subsequent analysis. For the higher wavenumber region, the spectra were cut in the region 2835–3000 cm⁻¹, baseline corrected and normalized to the CH₂ antisymmetric band (~2920 cm⁻¹), which has the maximum absorbance in this region. Other normalization methods such as amide I for the 800–1800 cm⁻¹ region or vector normalization for the entire spectra have also been tested and gave negligible changes in the results. The various biochemical parameters studied were glucose [12], phosphate levels [9,10,13], nucleic acid ratio [14], lipid–protein ratio [15,16].

3. Results

During the period of study, the patient was treated with a chemotherapy regimen which included Daunorubicin and Ara-C (Scheme 1). These drugs affect all WBCs and cause biochemical changes and apoptosis. Representative normalized spectra of WBC from the adult leukemia patient on several days of treatment are presented in Fig. 1a along with the average spectra of WBC from healthy persons (dashed line) for identification of the major changes in the spectra dur-
Fig. 1. (a) Representative spectra on different days of chemotherapy of an adult AML patient along with the average spectrum of healthy controls (dashed line). The important absorbance bands used in the analysis of the spectral data are marked. The spectra are the averages of five measurements at different sites on each sample. The inset displays the expanded spectra in the region 900–1000 cm$^{-1}$. (b) The higher wavenumber region between 2835 and 3000 cm$^{-1}$ normalized separately with respect to the 2925 cm$^{-1}$ band.

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The normal levels of glucose found in the WBCs as quantified by the spectral measurements in the patient during different days (solid squares) and those of the reference group of volunteers (open circles) are presented in Fig. 2a. The glucose was quantified from the second derivative spectra [12]. To confirm that this type of calibration was acceptable, the same methodology was verified on quantification of plasma glucose levels (Fig. 2b). Fig. 2b shows that there is a linear relation between the values obtained for glucose by blood biochemistry and those obtained from the spectral
measurements. Thus, the values shown in Fig. 2a give good representation of the amount of glucose (carbohydrate) in the WBCs during leukemia treatment. Fig. 2a indicates that the glucose level varies significantly in WBCs during treatment but it is mostly within the normal control limits with no specific trend during the entire duration of chemotherapy. This rules out the possibility of considering this parameter as an effective marker to monitor the changes.

Fig. 3a depicts the parameters derived from the symmetric vibrations of phosphates especially in nucleic acid (and also likely from proteins and lipids) absorbance normalized to amide II. The solid line represents the trend of the phosphate levels from WBC in adult. The data show an interesting pattern with a “U shape”. The open circles again represent the level from eight normal persons, which were used for control. The phosphate level falls in the initial days during induction of chemotherapy (up to day 7). A similar decrease in this ratio was reported earlier in apoptotic HL 60 cells [13]. Since the phosphates are mostly from the nucleic acids, this parameter shows similar trend in both cases. The increase in phosphate level in later phases after chemotherapy is not due to blasts in the peripheral blood but due to the return of normal levels of phosphates in WBCs which becomes comparable to that of the healthy group. A similar result was obtained from the intensity of the antisymmetric phosphate which indicates that these perhaps reflect changes in nucleic acids (Fig. 3b).

The WBC count of the patient also varied dramatically during the treatment and reached the normal levels as shown in Fig. 4. The normal range of WBC is between $4 \times 10^3$ and $11 \times 10^3$ cells/μl which was observed for the control population. However, this simple blood count should not be confused with the trends of phosphate depicted in Fig. 3a,b. We note that the final phosphate level relative to the initial is higher for an adult. However, the final levels are closer to the average values of the normal group represented by the open circles. This parameter has been earlier reported to increase or decrease depending on individual cases rendering it as inconsistent for a general follow-up in case of any leukemia [9,10]. Therefore, in spite of the diagnostic possibility, we do not advocate the utilization of this parameter alone for monitoring biochemistry of the WBCs.

The spectra were analyzed for additional possible biomarkers occurring due to absorbance form nucleic acids during chemotherapy. Fig. 5 shows the variation of the ratio of integrated absorbance due to amide II/amide I. This ratio has been indicated to represent the variation in nucleic acids [14]. It is observed that there are significant fluctuations in this ratio. However, at the end of the chemotherapy, the amide II/amide I ratio [9,10,14] reaches a normal level similar to the control group represented by the open circles. This ratio can be affected by several other parameters such as the amount of water in samples, the protein conformation and composition, presence/conformation of nucleic acids (factors that affect
Fig. 5. Variation in the amide II/amide I ratios during the treatment period obtained from the integrated absorbance in the region. The solid line and solid circles display the trend for the adult. The open circles are the values obtained for the healthy group. The values are the averages of five different measurements on each sample. The solid line intends only to guide the eyes and possibly represent the trends.

the amide I intensity) and thus the great variability from day to day can be expected.

The lipids in the plasma membrane are composed mainly of phospholipids that determine membrane stability, fluidity and membrane enzymatic activity. Thus, monitoring lipid absorbance of in WBCs is an important diagnostic parameter. The lipid–protein ratios were calculated using the intensities at 2852, 1740 and 1400 cm$^{-1}$ [15,16]. Fig. 6a,b represents the lipid–protein ratio for the same adult and two children as determined from the intensities at 2852, 1740 and 1400 cm$^{-1}$.

The lipid–protein ratio for the adult (Fig. 6a, solid line) decreases till day 7 when the chemotherapy is terminated and thereafter the ratio increases. The final values at the end of the treatment reach a normal level and may have implications for diagnosis based on FTIR-spectroscopy. It is also noted that the initial value on day zero (admittance day in the medical center) is slightly lower than the final value, which is similar to the healthy group. Therefore, this ratio maybe an important parameter for monitoring normalcy in WBCs population during chemotherapy. The adult patient was discharged after day 27 when he was found to be in clinical remission. Our data with FTIR also show that the spectra from WBCs were biochemically normal on this day. The return to normalcy for the adult occurred on a different time scale than reported for the children [9,10] as expected, since children normally respond faster (Fig. 6a). Furthermore, in this figure we can see that the patient’s WBCs biochemistry returns towards normal levels with progression of time. These data are in a good correlation with the clinical report of the patient and the clinical remission achieved.

Moreover, good correlation is observed with the medical report (includes possible indication for high fever) till recovery as seen from the return to the average of normal population on the day of discharge from hospital in clinical remission. Since the spectra are normalized to amide I or amide II which makes the protein constant, we can conclude that the lipids are the main cause for the changes in the lipid–protein ratio observed in Fig. 6a. Furthermore, a separation could also be achieved between the patient’s WBCs and the control group using the values calculated from the second derivative of the spectra (data not shown). The observations made in the case of children patients were also similar (Fig. 6a). It is interesting to note that the normalcy in the spectral parameters in children occurred earlier. Moreover, both children reached the normalcy earlier and were confirmed to have shown early and good response to the treatment.

Fig. 6. (a) Variation in the lipid–protein ratio as obtained form the absorbance at 2852 and 1400 cm$^{-1}$ after min–max normalization in the regions 2835–3000 cm$^{-1}$ and 400–1800 cm$^{-1}$ respectively for the adult AML patient and two ALL children patients used as test cases. The intensity at 1400 cm$^{-1}$ was used for the quantification of the proteins. The data points are the average of five different measurements on each sample. The open circles denote the values deduced for the healthy group. (b) The lipid–protein for the adult patient as calculated from the absorbance at 1740 cm$^{-1}$ after min–max normalization in the region 800–1800 cm$^{-1}$. The ‘solid line’ gives the trend. The ‘open circles’ denote the values deduced for the healthy group. The data points are the averages of five different measurements on each sample.
4. Discussion

FTIR-spectroscopy has been evaluated as a possible means for disease diagnosis with a promise at least for detection of abnormality in body cells and fluids [18,19]. Response to chemotherapy in leukemia patients is a critical factor to determine the treatment regimen and outlining the periods of induction and remission. It is also essential from the point of prescription of a maintenance period for the patient during "consolidation regimen". Thus, regular monitoring for presence of normal WBC, which is an indicator of the health status of the patient, is required.

Cells exhibit variability in FTIR spectral characteristics due to growth stages [3,15]. Thus, blasts when present in higher proportions in a preparation of WBC would have different spectra than the preparation of normal WBC. In the present study, we utilize this concept for detecting the return to normalcy of the WBC population, as a larger proportion of blasts in a random sample of WBC would confer different spectral features in the FTIR-spectra. As the proportion of the blasts comes down and WBC of normal composition begins to increase in the peripheral blood, the spectra of these WBC from patients must match the spectra obtained for healthy controls. Though in the adult by normal process of WBC counts (Fig. 4), there is no indication of leukemia, our corresponding FTIR measurements shows an abnormal spectra compared with the control. It is noteworthy that this patient had a history of myelodysplastic syndrome (MDS), which differs from leukemia only in the number of blasts in the bone marrow (normally an MDS patient shows symptoms of leukemia within 2 years). Due to changes in the blood count, a bone marrow examination was carried out which showed 30% of blast in the bone marrow. Further examination by immunophenotyping confirmed the diagnosis of acute myeloid leukemia (AML)-M1 subtype by the French American British (FAB) classification. In the present case study, the blast counts in peripheral blood were very low, with 5% blasts, and the WBC profile was comparable to the normal population.

It is observed from Fig. 1 that there are dynamic changes in the intensities of the normalized spectra, at various wavenumbers during chemotherapy, which correspond to biochemicals like carbohydrates, nucleic acids, lipids and other cellular components. One such wavenumber where intensity changes are prominent is the band around 966 cm$^{-1}$, which corresponds to absorbance due to nucleic acids and lipids [5,14,17]. The variation in this parameter (Fig. 1 inset) might be a result of the presence of blasts where nucleic acid signals are most likely to appear [2]. The total WBC counts for the adult patient decreased initially up to day 10 due to the effect of chemotherapy, became nearly stable till day 20 and then increased dramatically till it reached near normal levels of WBC count (4–11 × 10$^9$/L) on day 25–27 (Fig. 4). It is worth noting that the adult was discharged on day 27 when the clinical symptoms and routine tests indicated that he was normal (remission). The changes in the various spectral parameters are not in exact coherence with the measured numbers of WBCs indicating that it is the percentage of the blasts that is measured in the FTIR-MSP rather than the absolute quantity. However, the spectral features (Fig. 1a,b) themselves are indicative of presence of higher number of blasts.

Among the various parameters studied, the integrated phosphate absorbance normalized to amide II (Fig. 3a,b) and the lipid–protein ratio of the WBCs (Fig. 6a,b) calculated from two different band intensities representing the lipids [15,16] show promise as diagnostic parameters for monitoring the return to normalcy. This perhaps indicates the return of WBCs biochemistry to normal range implying a recovery of the immune system, reflected by the clinical improvement of the patient as shown in Scheme 1. In earlier works, the absorbance from the phosphate of nucleic acids was identified as a promising parameter for grading of malignancy [20]. In case of leukemia, the decrease in severity or the amount of blasts can represent different levels of cancer, the more serious leukemia being analogous to the more severe or higher grade of cancer. However, when the data were evaluated for the children, the parameter based on phosphate absorbance was not very dependable as it varied from patient to patient [9,10]. On the other hand, the lipid–protein ratio decreases initially and later increases, indicating apoptosis in the beginning and the presence of normal WBCs later, as would be expected for a person undergoing chemotherapy. The variation in the lipid profiles and their reactions with the body fluids during carcinogenesis have been well documented [21,22]. Thus, the variation in the lipid–protein parameter may mainly reflect the varying proportion of lipids between normal WBCs and blasts in leukemia. It is seen that this ratio is efficient for the adult (Fig. 6a) compared to children who recovered from leukemia by induction of chemotherapy [9,10]. The lipid–protein parameter reached a normal value after 25 days for the adult.

Parameters based on lipid absorbance for diagnosis are a possible utility of FTIR-spectroscopy as has been recently reported [23]. Using both protein and lipid absorbance would help to normalize the values for a more general application as there can be fluctuations in both these metabolites from patient to patient and the proposed ratio would help to eliminate individual variations or artifacts due to different baselines. It is also noteworthy that till the time of reporting of these observations (a period of 90 days after the adult was admitted with leukemia), there has been no relapse, indicating the return to normalcy. Furthermore, we see that the patient’s WBCs biochemistry returns towards normal values with progression in time, which is in good correlation with the clinical report of the patient and the clinical remission achieved. Thus, the lipid–protein ratio may be an effective way of monitoring return of a normal population of WBCs in leukemia patients and may possibly be developed as a rapid, objective, simple and supplementary to existing techniques. We must mention that the patient suffered from pancytopenia, which is a low WBC count, and thus normalcy at biochemical
level could be detected by FTIR from a small sample. The utilization of a different form of leukemia WBCs (from children) for validation of the results indicates the process maybe generalized (Fig. 6a). It also indicates that a normal population of WBCs after treatment in all patients is uniform in the spectral characteristics that were studied, and confirms the theory that each type of tissue or cells under normal conditions has a unique signature in the FTIR-spectra.

5. Conclusions

We evaluated biomarkers, derived from FTIR spectral measurements on WBCs from a leukemia patient, which could be suitable for monitoring return of normalcy. Among these, two parameters derived from lipid and protein absorbance showed promise and can possibly be used in tandem or as an additional follow-up technique to the conventional methods. A larger database to confirm these observations on AML and other types of leukemia is highly desirable.

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References


