FT-IR spectromicroscopy of mammalian cell cultures during necrosis and apoptosis induced by drugs

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Abstract. FT-IR is used in the field of biology and medicine to detect bimolecular changes in disordered cells and tissues. In this report, using FT-IR microscopy, we characterize changes in apoptotic and necrotic Jurkat cells with respect to normal cells. The analysis of deconvoluted regions of the FT-IR spectra showed significant differences compared to the controls in three spectral regions. In particular, the apoptotic cells were characterized by an increase in the absorption at 2925 cm^{-1} , due to the asymmetric CH_2 -stretching ($\nu_{\text{as}}\text{CH}_2$) of membrane lipids whereas the spectral areas ratio (A_{1654}/A_{1629}) of the amide I region indicated an increase in apoptotic cells of more α -helical structures with respect to of β -sheet content. Interestingly, apoptotic cells showed the appearance of a peak around 1743 cm^{-1} , $\nu(\text{C}=\text{O})$ assigned to acid ester. Because no other similar increase for lipid bands was observed, the increase of A_{1745} is not simply due to an increase in the number of lipid molecules or their density but could also be indicative as marker of apoptosis. These spectral changes were not observed in necrotic Jurkat cells.

Keywords: FT-IR micro-spectrometry, apoptosis, necrosis

1. Introduction

Techniques providing chemical information within living cells and tissues represent a very powerful tool for biomedical research directed toward the understanding of the causes of disease onset and on the properties and functions of diseased cells. Fourier Transformed Infrared spectromicroscopy (FT-IR-MC) has the potential to provide biochemical information without disturbing the biological sample. Moreover, it combines infrared spectroscopy, a sensitive analytical chemistry technique, with microscopy to enable detailed chemical analysis on a microscopic scale. Therefore, the FT-IR analysis of biological cells and tissue can determine whether cells or tissue are cancerous. Many important biological macromolecules, like nucleic acids [35], proteins [34] and lipids [19] have characteristic and well-defined IR-active vibrational modes thus, the analysis of IR spectra can detect, identify and quantify several molecular species within a biological sample. Under this aspects, FT-IR spectroscopy has been use to study variation in the conformation of biological macromolecules occurring between normal cells and cancer cells [1,2,4, 11,23,28,31]. In addition, FT-IR spectroscopy represents also an important tool for the comprehension

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of the interactions between living cells and their surrounding environments [37]. (The main advantages of this technique are simple, quick and economically viable).

In this study, changes in Jurkat cells during apoptosis or necrosis were studied using FT-IR microspectroscopy. With respect to necrosis, Jurkat cells showed significant conformational changes during apoptosis.

2. Materials and methods

2.1. Materials

RPMI 1640 medium and bovine fetal serum (FBS) were purchased from Lonza (Basel, Switzerland). Tissue culture plastic-ware was from Becton–Dickinson Labware (Franklin Lakes, NJ, USA). Propidium iodide was purchased from Sigma (Milan, Italy).

2.2. Cell growth and apoptosis

Human leukemic T cell line (Jurkat) obtained from American Type Tissue Collection (Rockville, MD, USA), was grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-glutamine at 37°C in a 5% CO₂ atmosphere up to a concentration of 5×10^5 cells/ml [15]. Apoptosis was induced treating cells with Etoposide phosphate (VP-16) (50 µM) or Cis-Platinum (Cis-P) (25 µM) [7,17]. After drug treatment, either at 8, 16, 24 and 32 h or at different drug concentration (1, 5, 10, 20, 30 µM for 48 h), cells were collected by centrifugation at 1500 rpm for 5 min, washed twice with 100 µl of PBS and resuspended in 10 µl PBS for FT-IR analysis. Necrosis of Jurkat cells [30] was induced at a concentration of 5×10^5 cells/ml by depriving the cell suspension of oxygen for 24, 48 and 96 h. Apoptosis or necrosis was evaluated by Propidium iodide and FACScan flow cytometer (Becton–Dickinson).

2.3. FT-IR analysis

In all experiments, 10 µl of cells suspension were layered on 3 mm ZnS window, dried for 2 min under a 60 watt white light (10 cm distant) and analyzed with a Nicolet 5700 equipped with a microscope Continuum (Thermo). For each sample, spectra (200 acquisitions) were collected always in transmission by using the microscope focusing windows set at 50×50 µm. Spectra were analyzed by using the Omnic software. Curve fit analysis of deconvoluted spectra was performed by using the OriginPro 7.5 software.

3. Results and discussion

3.1. Cellular changes in Jurkat cells

As reported in Table 1, the best condition for apoptosis was obtained by treating Jurkat cells with VP-16 for 32 h. Jurkat cells collected at different apoptotic stage or different necrotic stage were then analyzed by FT-IR microscopy. Representative FT-IR spectra of normal, apoptotic or necrotic cells are shown in Fig. 1. Spectra, normalized for the amide I peak, showed several absorbance changes. To evaluate these difference, the FT-IR spectra were analyzed in the regions corresponding to N–H stretching

Table 1
Apoptosis of Jurkat cells induced by Cis-platinum and VP-16

	Time (h)	Apoptosis (%)
No-treatment	0	3.08
Cis-P (25 μ M)	8	3.36
	16	11.64
	32	34.24
VP-16 (50 μ M)	8	6.40
	16	37.28
	32	66.66

Fig. 1. FT-IR spectra of Jurkat cells expressed as arbitrary units (a.u.). Approximately equal number of normal (continuous line), apoptotic (bold continuous line) or necrotic cells (dashed line) were spotted onto a ZnSe window, dried and analyzed under the IR microscope using $50 \times 50 \mu\text{m}$ window.

(3600–3000 cm^{-1}), C–H stretching (2800–3100 cm^{-1}), amide I (1750–1600 cm^{-1}), amide II (1600–1480 cm^{-1}), amide III (1300–1180 cm^{-1}) and DNA region (1300–800 cm^{-1}). The use of spectral areas derived from IR deconvolution rather than absolute optical density on selected absorbance was considered more informative [26].

3.2. The N–H stretching region

The deconvolution of the IR spectra in this region (Fig. 2) showed the presence of five peaks around the frequencies 3060, 3174, 3293, 3417 and 3534 cm^{-1} . The frequencies at 3293 and 3060 cm^{-1} can be generally assigned to the N–H stretching of proteins and nucleic acids (amide band A and B) whereas the band at 3417 cm^{-1} and 3174 cm^{-1} represent the N–H stretching bands of trans- and cis-ordered structures [5]. Evaluation of the absorbance peak area ratio A_{3293}/A_{3060} of the N–H stretching bands

Fig. 2. Deconvoluted IR spectra in the frequency region 3600–3000 cm^{-1} of Jurkat cells with enhancement factor 3.0 and bandwidth 250 cm^{-1} . (A) Normal cells; (B) cells after 32 h.

showed a progressive increase from control cells to cells treated with drugs at different times (Table 2a) and concentrations (Table 2b). The increase in the ratio was mainly due to the increase of the 3293 cm^{-1} peak area whereas the peak at 3060 cm^{-1} remained almost constant. This behaviour was also observed in Jurkat cells treated with rapamycin (control, 1.17 ± 0.06 ; rapamycin 32 h, 1.87 ± 0.04) and in KB cells treated with VP-16 (control, 1.35 ± 0.05 ; VP-16 32 h, 2.36 ± 0.04). We also analyzed the behaviour of Jurkat cells during necrosis. As reported in Table 2c, with respect to apoptotic cells, the A_{3293}/A_{3060} of the N–H stretching bands at 24, 48 and 96 h of necrosis remained almost unchanged. Because the intensity of the band at 3060 cm^{-1} is supposed to decrease with increasing dilution [18], this result might suggest that the apoptotic cell showed a more dense and close-packed structure with respect necrotic cells [5].

3.3. The C–H stretching region

FT-IR spectra in this region could furnish information regarding the oxidative damage of phospholipids. The deconvoluted spectra showed the presence of six peaks (Fig. 3). The band around 2850 and 2870 cm^{-1} is mainly due to the symmetric stretching of CH_2 ($\nu_s\text{CH}_2$) and CH_3 ($\nu_s\text{CH}_3$) groups of membrane lipids, respectively [9,25]. The band around 2925 and 2960 cm^{-1} is due to the asymmetric CH_2 - ($\nu_{as}\text{CH}_2$) and CH_3 -stretching ($\nu_{as}\text{CH}_3$) of methyl end-groups of membrane lipids, respectively, and also of the methyl amino acid side group of cellular proteins [9,25,29,38]. These marker bands are used to

Table 2a
(A_{3300}/A_{3075}) after treatment with different drugs

Control	VP-16 (50 μ M)			Cis-P (25 μ M)		
	8 h	16 h	32 h	8 h	16 h	32 h
1.48 ± 0.05	1.52 ± 0.06	1.70 ± 0.10	1.73 ± 0.10	1.36 ± 0.04	1.44 ± 0.06	1.52 ± 0.10

Table 2b
(A_{3300}/A_{3075}) after treatment at increasing concentration of VP-16

Control	VP-16 concentration				
	1 μ M	5 μ M	10 μ M	20 μ M	30 μ M
1.68 ± 0.06	1.69 ± 0.10	1.76 ± 0.10	1.82 ± 0.04	1.83 ± 0.06	1.87 ± 0.10

Table 2c
(A_{3300}/A_{3075}) at increasing time of oxygen deprivation

Control	24 h	48 h	96 h
2.0 ± 0.1	1.88 ± 0.10	2.12 ± 0.10	1.98 ± 0.10

Fig. 3. Deconvoluted IR spectra in the frequency region 3000–2800 cm^{-1} of Jurkat cells with enhancement factor 3.0 and bandwidth 30 cm^{-1} . (A) Normal cells; (B) cells after 32 h treatment with 50 μ M VP-16. Deconvoluted spectra (continuous line), spectral curve-fitting (dashed line).

determine the membrane lipid content [31]. Curve fitting analysis of the absorption areas showed during apoptosis an increase with respect to untreated cells of $\nu=\text{CH}$ (3010 cm^{-1}) (from 6.1 to 7.3%) and $\nu_{\text{as}}\text{CH}_2$ (from 28.8 to 30.2%) and a slight decrease of the $\nu_{\text{as}}\text{CH}_3$ peak area (from 23.3 to 21.9%). This behaviour was also concomitant to the increase of the $\nu_s\text{CH}_2$ (from 17.5 to 19.2%) and the decrease of $\nu_s\text{CH}_3$ (from 15.2 to 13.2%) peak areas. The increase in the A_{2852} indicated a higher membrane lipid content in Jurkat cells during apoptosis compared to that of untreated cells [41]. We also observed a progressive increase of the $\nu=(\text{CH})/\nu_{\text{as}}(\text{CH}_3)$ (A_{3010}/A_{2960}) and $\nu_{\text{as}}(\text{CH}_2)/\nu_{\text{as}}(\text{CH}_3)$ (A_{2925}/A_{2960}) peak ratio from untreated cells (0.26 ± 0.03 and 1.23 ± 0.10 , respectively) to cells treated for 32 h with VP-16 (0.35 ± 0.04 and 1.31 ± 0.08 , respectively). Comparable result was also observed in the case of Jurkat cells treated with Cis-P. In the case of necrotic Jurkat cells, the analysis of deconvoluted spectra at 0 (control), 24, 48 and 96 h did not show substantial differences in both peak area and ratio. In fact, the $\nu=(\text{CH})/\nu_{\text{as}}(\text{CH}_3)$ and $\nu_{\text{as}}(\text{CH}_2)/\nu_{\text{as}}(\text{CH}_3)$ values were 0.50, 0.48, 0.58, 0.48 and 1.27, 1.17, 1.19, 1.17, respectively. The increase of CH_2/CH_3 ratio in Jurkat apoptotic cells was due to a slightly decrease of the $\nu_{\text{as}}(\text{CH}_3)$ with respect to the $\nu_{\text{as}}(\text{CH}_2)$. From this data, it appeared that there was not a direct correlation between unsaturation and saturation level of membrane phospholipids as observed instead in the case of erythrocytes oxidative stress [26]. Also in the case of apoptotic HL60 cells, no significant changes in the CH_2/CH_3 ratio was observed [10]. These findings probably indicated that with respect to oxidative stress [26], fatty acid metabolism, evaluated by change in the $\nu_{\text{as}}(\text{CH}_3)$ absorbance [26], was changing in apoptotic Jurkat cells. It was reported that the tendency in the increase in the CH_2/CH_3 absorbance ratio might depends by cell growth conditions [3,14,20,21,33]. Because apoptosis lead to cell shrinkage [16], a decrease in the cellular volume should correspond to a relative increase in the surface area. This may cause an increase of the phospholipids fraction in the biomass, corresponding to a major enrichment in CH_2 relative to CH_3 groups [10].

3.4. The amide I region

The amide I region is often used in the study of membrane-protein structures since this region represents the most suitable probe to evaluate differences among the secondary structures of proteins and peptides [12]. The deconvolution spectra (Fig. 4) showed the presence of seven major $\nu(\text{C}=\text{O})$ bands around 1594 , 1629 , 1654 , 1675 , 1691 , 1714 and 1745 cm^{-1} corresponding to amine, parallel β -strand, α -helix, parallel β -strand, anti-parallel β -strand, lipid ester and acid ester, respectively [27]. Curve fit Analysis of the deconvoluted curves showed that the α -helix content increased from control cell (28%) to cell treated with VP-16 for 32 h (33.5%) whereas the peaks of β -strand at 1675 cm^{-1} showed a reduction (from 15.5 to 14.0%) [13]. The A_{1654}/A_{1629} furnishes a measure of the α -helix and β -strand domain of proteins [5]. Here, we observed an increase of the ratio (control cell, 0.43 ± 0.03 ; Cis-P 8 h, 1.74 ± 0.06 ; Cis-P 16 h, 1.67 ± 0.06 ; Cis-P 32 h, 1.22 ± 0.05). The same evaluation made for necrotic Jurkat cells showed a tendency to remain constant or to a reduction (control, 1.10 ± 0.04 ; 24 h, 1.15 ± 0.04 ; 48 h, 0.95 ± 0.03 ; 96 h, 1.09 ± 0.04) [32]. It could be possible that the conversion from α -helix to β -sheet structure could be due to lipid-auto-oxidation products that probably deformed the protein's secondary structure from α -helix to β -sheet [39].

In addition, we also observed a strong increase of the peak at 1745 cm^{-1} (from about 6.5 to 10.5%), usually associated with the non-hydrogen bonded ester carbonyl $\text{C}=\text{O}$ stretching mode within phospholipids [24], that was even more evident during treatment of Jurkat cell with increasing concentration of VP-16 (from 5.0% control up to 12.8% at $30\text{ }\mu\text{M}$ VP-16) and a peak at 1716 cm^{-1} , associated with hydrogen bonded $\text{C}=\text{O}$ groups [6], that instead showed a decrease (from 9.8 control to 6.4%). These

Fig. 4. Deconvoluted IR spectra in the frequency region $1600\text{--}1760\text{ cm}^{-1}$ of Jurkat cells with enhancement factor 3.0 and bandwidth 40 cm^{-1} . Spectra were taken at different times of treatment of Jurkat cells with $50\text{ }\mu\text{M}$ VP-16.

variations were not observed in necrotic cell. In fact, the absorbance peak area at 1745 cm^{-1} ($\sim 9.1\%$ control; 9.5% at 24 h; 9.8% at 48 h; 9.7% at 96 h) and 1716 cm^{-1} ($\sim 8.9\%$ control; 8.7% at 24 h; 8.5% at 48 h; 8.9% at 96 h) remained almost unchanged. In the latter case, an increase in the peak intensity (1725 cm^{-1}) [6] was instead observed in necrotic cells. Holman et al., 2003 [13] and Jamin et al., 1998 [14] reported a similar increase in the case of IMR-90 cells that had lost membrane integrity [40]. This finding indicated that the C=O ester carbonyl groups of lipids in apoptotic cells are becoming predominantly non-hydrogen bonded [13] which would be indicative of oxidative damage. In fact, among other factors, apoptosis is associated with increased oxidative damage [22].

3.5. The amide II region

The amide II region $\delta(\text{N-H})$, after deconvolution, showed the presence of absorption bands at 1506 , 1521 , 1540 (predominantly β -sheet), 1558 (predominantly α -helix), and 1575 cm^{-1} (Fig. 5). This region however, is not as sensible to conformational changes as instead for the amide I bands [24]. We found that the peak area ratio A_{1558}/A_{1540} slightly increased after treatment of the cells with drugs (0.58 ± 0.03 for normal cell and 0.58 ± 0.03 , 0.68 ± 0.03 and 0.69 ± 0.03 for cell treated with VP-16 at 8, 16 and 32 h, or 0.62 ± 0.03 for cell treated with Cis-P at 8 and 16 h, respectively), thus suggesting an increase in the absorption of α -helix. On the contrary, in the case of necrotic cell, the value of the A_{1558}/A_{1540} ratio was 1.05 ± 0.05 , 1.03 ± 0.05 and 0.98 ± 0.03 at 24, 48 and 96 h, respectively. Evaluation

Fig. 5. Deconvoluted IR spectra in the frequency region 1600–1480 cm^{-1} of Jurkat cells with enhancement factor 3.0 and bandwidth 30 cm^{-1} . (A) Normal cells; (B) cells after 32 h treatment with 50 μM VP-16. Deconvoluted spectra (continuous line), spectral curve-fitting (dashed line).

of amide I–amide II peak area ratio (A_{1654}/A_{1540}) (α -helix/ β -sheet), which is often used to identify conformational changes of membrane proteins [36], showed a slight increase (0.98 ± 0.04 in control cell and 0.98 ± 0.04 , 1.04 ± 0.05 and 1.14 ± 0.06 for cell treated with VP-16 at 8, 16 and 32 h, or 1.14 ± 0.06 for cell treated with Cis-P at 8 and 16 h, respectively). The same evaluation made for necrotic cells showed an almost constant ratio (0.95 ± 0.03 , 0.98 ± 0.04 and 1.00 ± 0.04 at 24, 48 and 96 h, respectively).

3.6. The amide III region

Spectra deconvolution of the amide III region shows the presence of three peaks at 1212, 1237 and 1284 cm^{-1} (Fig. 6). The major band at 1237 cm^{-1} represents also the asymmetric stretching of phosphodiester bond ($\nu_{\text{as}}\text{PO}_2^-$) of nucleic acid. The bands at 1212 and 1284 cm^{-1} of low intensity have been assigned to vibration modes of collagen proteins. Evaluation of peak area showed a slight increase of the peaks at 1212 and 1284 cm^{-1} with respect to control cells that was associated to a decrease of the peak area at 1237 cm^{-1} . The A_{1657}/A_{1212} and A_{1657}/A_{1284} indicate the collagen content relative to total content of protein [5]. In the case of Jurkat cells, no strong differences in apoptotic or necrotic cells were found as instead observed in tumoral tissues [5]. In particular, in Jurkat cells treated with VP-16, both ratios showed a very small increase (control, 0.97 ± 0.04 ; VP-16 8 h, 1.06 ± 0.04 ; VP-16 32 h, 1.07 ± 0.04 ; VP-16 32 h, 1.11 ± 0.05 and control, 1.11 ± 0.05 ; VP-16 8 h, 1.15 ± 0.06 ; VP-16 32 h,

Fig. 6. Deconvoluted IR spectra in the frequency region 1180–1300 cm^{-1} of Jurkat cells with enhancement factor 3.0 and bandwidth 30 cm^{-1} . (A) Normal cells; (B) cells after 32 h treatment with 50 μM VP-16. Deconvoluted spectra (continuous line), spectral curve-fitting (dashed line).

1.20 ± 0.06 ; VP-16 32 h, 1.19 ± 0.06 , respectively) whereas, in necrotic cells the values at 24, 48 and 96 h were 0.87 ± 0.03 ; 0.78 ± 0.03 ; 0.88 ± 0.03 and 0.97 ± 0.04 ; 0.96 ± 0.04 ; 0.98 ± 0.04 , respectively.

3.7. The DNA region

Analysis of the deconvoluted spectra of this region showed the presence of several peaks (Fig. 7). The bands around 965, 1087 and 1240 cm^{-1} are considered DNA bands. The 965 cm^{-1} band corresponds to C–C/C–O stretching of deoxyribose and phosphate of DNA [2]. The band around 1087 cm^{-1} derives from the symmetric PO_2^- ($\nu_s\text{PO}_2^-$) whereas the band around 1240 cm^{-1} originates from the asymmetric PO_2^- ($\nu_{as}\text{PO}_2^-$) of the phosphate group of DNA [38]. The peak area ratio A_{1087}/A_{1540} is often used to detect change of the DNA/protein content in the cell [35]. Compared to normal cells, we observed an increase of the ratio in Jurkat cells treated with VP-16 (control 0.37 ± 0.03 vs. 0.41 ± 0.04 , 0.46 ± 0.04 and 0.47 ± 0.04 in the case of cell treated with VP-16 at 8, 16 and 32 h, respectively), indicating an increase in the DNA content of apoptotic cells [2,36]. These changes were not observed in necrotic cells in fact the A_{1087}/A_{1540} value was 0.47 ± 0.03 ; 0.48 ± 0.04 and 0.45 ± 0.04 at 24, 48 and 96 h, respectively.

Fig. 7. Deconvoluted IR spectra in the frequency region 1300–800 cm^{-1} of Jurkat cells with enhancement factor 3.0 and bandwidth 30 cm^{-1} . (A) Normal cells; (B) cells after 32 h treatment with 50 μM VP-16. Deconvoluted spectra (continuous line), spectral curve-fitting (dashed line).

4. Conclusion

Cell death could be classified into two categories: (a) apoptosis, representing “active” programmed cell death, (b) necrosis, representing “passive” cell death. Both are distinguished by well defined morphological and biochemical features. Necrosis is characterized by cell swelling, disruption and rapid disintegration of the cell membrane [8] whereas in apoptosis, the cells undergo nuclear and cytoplasmic shrinkage, chromatin condensation and fragmentation, and finally the cells are broken into multiple membrane surrounded bodies (apoptotic bodies) [8]. In this work, we have compared FT-IR features of Jurkat cells during induced apoptosis and necrosis and some information were derived from the analysis of the spectra:

- (A) In contrast to necrotic cells, the apoptotic cells were characterized by an increase in the CH_2 absorption [32].
- (B) The protein distribution in apoptotic cells indicates the presence of more α -helical structures, whereas proteins in necrotic cells become rapidly more and more aggregated [39]. In fact, the A_{1654}/A_{1629} observed in H1355 necrotic cells was even indicative of an increase of β -sheet content (nec 48 h).

- (C) In apoptotic cells there was the appearance of a peak around 1743 cm^{-1} . Because no other similar increase for lipid bands was observed, the increase of A_{1745} is not simply due to an increase in the number of lipid molecules or their density but could also be indicative as marker of apoptosis.

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