FAST TRACK

Serum cytochrome C indicates in vivo apoptosis and can serve as a prognostic marker during cancer therapy

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Despite significant progress in cancer therapy, the outcome of the treatment is often unfavorable. Better treatment monitoring would not only allow an individual more effective, patient-adapted therapy, but also it would eliminate some of the side effects. Using a cytochrome c ELISA that was modified to increase sensitivity, we demonstrate that serum cytochrome c is a sensitive apoptotic marker in vivo reflecting therapy-induced cell death burden. Furthermore, increased serum cytochrome c level is a negative prognostic marker. Cancer patients whose serum cytochrome c level was normal 3 years ago have a twice as high probability to be still alive, as judged from sera samples collected for 3 years, analyzed recently and matched with survival data. Moreover, we show that serum cytochrome c and serum LDH activity reflect different stages and different forms of cell death. Cellular cytochrome c release is specific for apoptosis, whereas increased LDH activity is an indicator of (secondary) necrosis. Whereas cytochrome c ELISA activity is an indicator of (secondary) necrosis, whereas LDH activity reflects the "global" degree of cell death over a period of time, the sensitive cytochrome c-based method allows confirmation of the individual cancer therapy-induced and spontaneous cell death events. The combination of cytochrome c with tissue-specific markers may provide the foundation for precise monitoring of apoptosis in vivo, by "lab-on-the-chip" technology.

Key words: apoptosis; bystander effect; LDH; prognostic factor; treatment monitoring; cytochrome c

Cancer therapy protocols mostly lack patient-oriented individualization because clinicians lack rapid and precise therapy-monitoring methods. The therapy success is usually assessed by estimation of the (decrease of) tumor burden or by the improvement of overall clinical conditions. Chemotherapy kills cells by apoptosis rather than by necrosis. Apoptosis, or programmed cell death (PCD), also commonly occurs in the development and a number of (patho)physiologic conditions. In contrast, necrosis is a more passive process that mostly arises when cells have been severely damaged by noxious insults. Apoptotic signals converge either on death receptor-triggered caspase cascades or on the mitochondria/apoptosome pathway. In the latter case, the initial and crucial event is the release of cytochrome c from mitochondria into the cytosol, which can be triggered by diverse apoptotic stimuli including anticancer drugs and irradiation. Cytosolic cytochrome c together with dATP binds to the apoptosis regulator Apaf-1, thus leading to the formation of the apoptosome and the initiation of the proteolytic, caspase death cascade.

Members of the Bcl-2 family play a key role in regulation of cytochrome c release. The family is composed of both antiapoptotic and proapoptotic proteins. It is widely accepted that the antiapoptotic Bcl-2 molecules function to prevent the release of cytochrome c and other proapoptotic molecules from mitochondria; they may also counteract the proapoptotic Bcl-2 family members. There are 2 subgroups of proapoptotic Bcl-2 molecules. Members of one subgroup, best represented by Bax and Bak, have 2 or 3 BH regions and appear to be structurally similar to their prosurvival relatives. The second subgroup of proapoptotic Bcl-2-related proteins, (e.g., Bax, Bad, Bid, Bim, PUMA, NOXA) share only the short BH3 region. BH3-only proteins appear to sense stimuli that cause cellular stress and initiate the death cascade. Proapoptotic Bax and Bak are essential for cell killing governed by BH3-only proteins, and this form of cell death is antagonized by overexpression of Bcl-2.

We and others have previously described that cytochrome c is not only released from mitochondria, but furthermore it leaves the cell and can be considered as a novel biochemical indicator of apoptosis. In our study, using an improved, highly sensitive, cytochrome c ELISA, we monitored cytochrome c in the extracellular medium of apoptotic cells and in the serum of cancer patients. In a significant number of patients, the serum cytochrome c levels were high or increased upon the onset of chemotherapy and decreased gradually during remission induction. Furthermore, an increased serum cytochrome c level appears to be a negative prognostic marker. Patients with an elevated overall cytochrome c level prior to and during therapy have about a 2-fold decreased chance of 3-year survival compared to those ones with a comparable to normal cytochrome c level. A high serum cytochrome c level probably indicates a high tumor load at least in some patients.

Material and Methods

Material and cell culture

All cell lines were grown in 5% CO₂ at 37°C using RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics (GIBCO, Eggenstein, Germany). Oligomycin, etoposide and doxorubicin were purchased from Sigma (Deisenhofen, Germany) and staurosporine from Alexis (San Diego, CA). All other chemicals were from Merck KG (Darmstadt, Germany) or Roth (Karlsruhe, Germany).

Serum sample processing, cell extracts, immunoprecipitation and Western blotting

Our study was approved by the University’s Ethical Board. Sera (4 ml) from 21 tumor patients (16 males, 5 females, age 22–79) were analyzed. The average age was 49.7 years. Further information characterizing the cohort is indicated in Table 1. Control samples were obtained from age and sex-matched laboratory personnel and healthy volunteers. All samples were preclarified by centrifugation at 10,000g, 4°C for 15 min. Cell lysis, immunopre-
TABLE I – PROFILE OF PATIENTS PARTICIPATING IN THE STUDY

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patient no.</th>
<th>Therapy protocol1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute myeloid leukaemia (AML)</td>
<td>1, 8</td>
<td>Maintenance/postremission therapy (M-1: cytosine arabinoside, daunorubicin)2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>TAD (thioguanine, cytosine arabinoside, daunorubicin)2,3</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Cytosine arabinoside2,3</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>HAM (high-dose cytosine arabinoside, mitoxantrone)4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ICE (idarubicine, cytosine arabinoside, etoposide)2,3</td>
</tr>
<tr>
<td>Acute lymphoblastic leukaemia (ALL)</td>
<td>16</td>
<td>GMALL (for ALL-relapse)2,3</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma (NHL)</td>
<td>5, 19</td>
<td>GMALL (for elderly patient)7,8</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Pretreatment (100 mg prednisolone, 1 mg vincristine), CHOP (cyclophosphamide, vincristine, prednisolone, doxorubicin)2,9</td>
</tr>
<tr>
<td>Hodgkin’s diseases (HD)</td>
<td>17, 21</td>
<td>CHOP (cyclophosphamide, vincristine, prednisolone, doxorubicin)2,9</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>20</td>
<td>ICE (ifosfamide, carboplatin, etoposide)11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>DHAP (dexamethasone, high-dose cytosine arabinoside, cisplatin)2,12</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>DHAP (dexamethasone, high-dose cytosine arabinoside, cisplatin)2,12</td>
</tr>
<tr>
<td>Breast cancer (Carcinosarcoma)</td>
<td>6</td>
<td>Dexamethasone, 40 mg; days 1–4</td>
</tr>
<tr>
<td>Germ-cell tumor (Seminoma)</td>
<td>15</td>
<td>ID (idarubicin, dexamethasone)13</td>
</tr>
<tr>
<td>Nonsmall cell lung carcinoma (NSCLC)</td>
<td>4</td>
<td>Epirubicin, paclitaxel1</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>PEI1</td>
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</tbody>
</table>


Results
Optimization of cytochrome c ELISA
An early event in apoptosis induced by death receptor-independent stimuli is the translocation of cytochrome c into the cytosol.18,19 We and others have shown that cytochrome c is not only released from the mitochondria upon apoptosis induction (Fig. 1a), but furthermore it leaves the cell and can even be detected in the serum of cancer patients upon chemotherapy.11,12 A previously used immunoprecipitation-based method of cytochrome c detection was labor intensive, error prone and impractical due to the loss of variable amounts of cytochrome c during the pre-clearance with protein-G (see also Fig. 1d,e). In an attempt to circumvent these problems, we sought a different method. Thus, we have modified a commercially available cytochrome c ELISA to increase its sensitivity and to improve serum compatibility. This allowed quantification of cytochrome c not only in the cell medium but also in the patient’s sera even after several dilutions (Fig. 1b,c). Sera dilutions up to 20 times were routinely carried out to eliminate variations caused by residual proteins. Pre-clearance of sera with protein-G sepharose, an alternative approach tested, caused a loss of variable amounts of cytochrome c (Fig. 1d), therefore it was not practiced. The mechanism(s) that contribute to the loss of cytochrome c during the pre-clearance of sera with protein-G sepharose was investigated (Fig. 1e). The pre-clearance-related decrease of cytochrome c was observed not only in primary cancer patient sera (Fig. 1e, compare lanes 1 and 2) but also in sera samples already pre-cleared with protein-G sepharose and subsequently supplemented with 50 ng cytochrome c (Fig. 1e, compare lanes 3 and 4). We concluded from this experiment that the nonspecific pre-clearance-related cytochrome c loss was primarily related to its nonspecific binding to protein-G sepharose, although presence of anti-cytochrome c antibodies in cancer-patient sera cannot be fully ruled out.
Different release kinetics of cytochrome c and LDH from apoptotic and necrotic cells

Apoptotic stimuli induce necrosis in cells depleted of ATP. Both death processes rely on different physiologic mechanisms and lead to different responses from the surrounding tissues. Because cytochrome c and LDH are released from dying cells and can serve as cell death markers, we compared the release kinetics by both forms of cell death in the same cell line and upon identical stimulus. ATP was depleted by the blocking of ATP-synthesis by oligomycin and keeping cells in a glucose-free medium 1 hr prior to the start of the experiment. Cytochrome c is released from apoptotic but not from necrotic cells (Fig. F2a). LDH release is observed in both types of cell death but with different intensity and kinetics. Although apoptotic cells start releasing some LDH from 8–12 hr after stimulation, a much stronger LDH release is observed during necrosis but at later time points, becoming promi-
5–7 times more sensitive as an in vitro (Fig. 2 by a flow cytometry measurement of the hypodiploid nuclei we measured cytochrome c content in these sera-samples with a Prognostic and diagnostic value of serum cytochrome c level that differentially describe cell death compared to the LDH.12

Figure 3 – Monitoring of serum cytochrome c level and serum LDH activity by cancer patients undergoing chemotherapy. Serum cytochrome c level and serum LDH activity were monitored on daily bases in cancer patients treated in 1999–2000. Day 0 is the day prior to the beginning of chemotherapy.

Serum cytochrome c level and serum LDH activity; parameters that differentially describe cell death in vivo

Inspired by differential kinetics of cytochrome c and LDH release from dying cells, we compared both parameters in vivo by examining the sera of cancer patients under chemotherapy. Both parameters show roughly parallel kinetics (Fig. 3); however, the serum cytochrome c appears to be a more sensitive indicator of individual cell death events, vigorously responding to incidents of increased apoptosis induced by chemotherapy (individual patient diagrams, Fig. 3). This becomes most prominent upon the analysis of both parameters by patient 3 (and later by the analysis of patients 16 and 18, Fig. 4b). Patient 3 has been suffering from an AML. During the first 3 days of treatment, the patient developed a mild form of tumor lysis syndrome. His leukocytosis decreased from 60,000/μl to 57,600/μl, which was associated with an increased hyperfibrinolysis, a slight increase of serum LDH activity (Fig. 3), an increase of C-reactive protein and an increase of phosphate and uric acid. Thus, the comparison of kinetics for both parameters clearly displays that cytochrome c is a much more sensitive indicator of apoptosis-induced therapeutic effects. A high level of anti-apoptotic Bcl-2 family members would prevent a positive feedback from the bystander effect.11 This observation corroborates well with the serum cytochrome c level measured by the patients 11 (AML) and 15 (multiple myeloma). Despite high initial tumor load, patient 11 achieved complete remission and patient 15 partial remission. Alternatively, the high serum cytochrome c level observed in these patients was due to sustained killing of neoplastic cells by the therapy. We favour the latter explanation because the contribution of cytochrome c to a bystander effect has not been fully elucidated. While the future research of our lab focuses on its possible immunomodulatory role (prevention of inflammatory response induction by apoptotic cells), at least in the neural tissue, cytochrome c released from dying cells contributes to the bystander effect.11 This observation corroborates well with the serum cytochrome c level measured by the patients 11 (AML) and 15 (multiple myeloma). Despite high initial tumor load, patient 11 achieved complete remission and patient 15 partial remission. Additionally, the high serum cytochrome c level observed in these patients was due to sustained killing of neoplastic cells by the therapy. We favour the latter explanation because the contribution of cytochrome c to a bystander effect has not been demonstrated yet in haematologic malignancies. Still, both possibilities are not mutually exclusive. Thus, bystander effect would lead to increased apoptosis and further release of cytochrome c. Such cytochrome c-dependent therapeutic effects would rely on the intactness of the mitochondrial/apoptosome death pathway. A high level of anti-apoptotic Bcl-2 family members would prevent a positive feedback that facilitates the release of cytochrome c from mitochondria.4,10 Similarly, overexpression of Inhibitor of Apoptosis Proteins, particularly XIAP,21,22 as well as a decreased level of their modulators Smac and OMI/HtrA2, would interfere with a cytochrome c–mediated bystander effect.23,24 Furthermore, it has been described recently that cytochrome c may activate K⁺-channels and thus

Prognostic and diagnostic value of serum cytochrome c level

We have been daily collecting sera from 21 patients under chemotherapy due to malignancies in 1999/2000. Three years later, we measured cytochrome c content in these sera-samples with a highly sensitive ELISA and correlated it with the survival data (Fig. 4a–c). Of 21 patients included in our study, 8 were still alive 3 years later (Fig. 4a–d). Six of them (75%) had a generally normal serum cytochrome c level during the treatment 3 years ago. The cytochrome c level ≤25 ng/ml was defined as normal, based on the measurement of serum cytochrome c by 11 volunteers. Figure 4a shows sera cytochrome c kinetics during treatment in 2000, from 11 patients who have since died. Patients 16 (ALL) and 18 (HD) show a strong increase of cytochrome c level around the 8th and 9th day of treatment. The finding correlates well with other biochemical and clinical parameters. The strong increase of cytochrome c by patient 16 was most likely due to chemotherapy-related liver toxicity. A strong increase of liver enzymes like ALT, AST and γ-GT have been observed at the same time. Similar observations have been recently published by others.20 Patient 18 developed a strong, chemotherapy-related mucositis on the 8th day of chemotherapy. Thus, the sharp increase of serum cytochrome c level by the patient was likely due to the inflammatory damage of the mucosa. Figure 4c shows patients with a high serum cytochrome c level regardless of the 3-year survival. Common to these 4 patients was a high tumor load. For example, patient 20 had an aggressive, relapsed stadium-IV lymphoma with diffuse infiltrations in various tissues. He died later that year due to the tumor progression. The tumor load in patient 11, with the 2nd highest overall cytochrome c level, was also very high (>60,000 AML-cells/μl), and aggressive chemotherapy was not able to induce a full remission. Fortunately, following chemotherapy cycles were more effective, so complete remission was achieved in 2002. Figure 4d,e shows the average cytochrome c level by deceased and surviving patients. In Figure 4d, data on all patients has been included. In both groups (surviving and deceased), single patients with a much higher serum cytochrome c level compared to the remaining group members existed. Therefore, in Figure 4e, we repeated the analysis after exclusion of these single patients from both groups (patients 11 and 20). Thus, albeit of the approach used, in all surviving patients show on average a lower cytochrome c level. The *-marked significant peak of cytochrome c (Fig. 4e) is caused by the sharp increase of the serum cytochrome c level at days 8 and 9 by patients 16 and 18 (see above discussion of Fig. 4b).

Discussion

The apoptosis-specific release of cytochrome c to extracellular space has been described previously in vitro and in vivo upon chemotherapy,11,12 but the biologic significance of it has not been fully elucidated. While the future research of our lab focuses on its possible immunomodulatory role (reduction of inflammatory response induction by apoptotic cells), at least in the neural tissue, cytochrome c released from dying cells contributes to the bystander effect.11 This observation corroborates well with the serum cytochrome c level measured by the patients 11 (AML) and 15 (multiple myeloma). Despite high initial tumor load, patient 11 achieved complete remission and patient 15 partial remission. Additionally, the high serum cytochrome c level observed in these patients was due to sustained killing of neoplastic cells by the therapy. We favour the latter explanation because the contribution of cytochrome c to a bystander effect has not been demonstrated yet in haematologic malignancies. Still, both possibilities are not mutually exclusive. Thus, bystander effect would lead to increased apoptosis and further release of cytochrome c. Such cytochrome c-dependent therapeutic effects would rely on the intactness of the mitochondrial/apoptosome death pathway. A high level of anti-apoptotic Bcl-2 family members would prevent a positive feedback that facilitates the release of cytochrome c from mitochondria.4,10 Similarly, overexpression of Inhibitor of Apoptosis Proteins, particularly XIAP,21,22 as well as a decreased level of their modulators Smac and OMI/HtrA2, would interfere with a cytochrome c–mediated bystander effect.23,24 Furthermore, it has been described recently that cytochrome c may activate K⁺-channels and thus
directly contribute to apoptosis-related cell shrinkage. Nevertheless, the role of cytochrome c as a mediator of the bystander effect still awaits further examination.

Serum cytochrome c is a precise indicator of cell death episodes in vivo (Fig. 3). It is released to the extracellular medium earlier than LDH and in larger quantities (Fig. 2). Given 10-times difference in molecular mass, cytochrome c (approx. 14 kDa) is much more likely to be cleared through the kidneys than the LDH (tetramer's mass: approx. 140 kDa). Therefore, its serum level changes dynamically and is directly linked to the actual ongoing cell death events. The lack of increase of serum cytochrome c level upon the start of therapy by some patients (Fig. 3 and 4a–c) may have been missed due to a predicted rapid kidney clearance.

A more frequent than just daily assessment of serum cytochrome c and/or combination with measurements done in urine would likely provide a more precise insight into therapy-induced cell death.

Because both molecules are located in different cellular compartments, cytochrome c in the mitochondrial intermembrane compartment and LDH in the cytoplasm, their release will be governed by different cellular processes. The translocation of cytochrome c to the cytoplasm is a prerequisite for the initiation of the apoptosome-dependent apoptotic cascade, therefore, serum cytochrome c level is the indicator of apoptotic rather than necrotic cell death. LDH resides in the cytoplasm and is separated from the extracellular space by a single lipid (cellular) membrane. Also, the
release mechanisms of both molecules may differ significantly due to a large difference in size. It is unlikely that cytochrome c is released from cells by a simple cell lysis because a release of LDH occurred at later time points (Fig. 2). A number of proteins, such as HIV-Tat, thioredoxin, interleukin 1β and basic fibroblast growth factor, which lack a signal peptide, are exported by alternative, not well-defined pathways.26 For example, death ligands, such as CD95L and TRAIL, are stored in microvesicles that are released on demand, upon activation or apoptosis.27,28 However, pharmacologic inhibitor experiments (data not shown) argue against a related release mechanism by cytochrome c.

Prognostic markers help to predict the outcome of disease and thus to aid in selecting high-risk patients for more aggressive and/or experimental therapy. Several markers exist that are mostly useful for single diseases or a cluster of malignancies.29-33 The usefulness of these markers is mostly limited to a group of diseases at best, therefore, a typical clinical laboratory would need to be furnished with hundreds of tests to cover the broad spectrum of malignancies typically found in the clinical practice. Cytochrome c certainly covers all malignancies since no cancer devoid of mitochondria exists. Its broad spectrum is achieved for the price of the selectivity. The increase of serum cytochrome c indicates, with a good degree of precision, the increase of apoptosis in vivo (see above discussion of patients 3, 16 and 18). Some of the serum cytochrome c may also be released from healthy tissue because current chemotherapy is also considerably toxic to some types of cells. Correlation of cytochrome c values with other clinical findings indicated that by patients 16 and 18 that significant hepatotoxicity and inflammatory mucosal damage, respectively, contributed to spikes of high cytochrome c level (Fig. 4b). Thus, it is the combination of data on cytochrome c (indicator of apoptosis) with other markers (e.g., increased enzymatic activity typical for liver cells, see above, patient 16) that provide a clearer picture of the patient’s response to the applied treatment. As efforts to develop “lab-on-the-chip” technology are on the way, the combination of cytochrome c as an apoptotic marker together with indicators specific for various tissues and/or developmental stages will one day allow precise detection and localization of cell death in vivo.34-36

In summary, we show that the serum cytochrome c is a sensitive apoptotic indicator in vivo that favorably compares with the LDH. Furthermore, high-serum cytochrome c appears to be a negative prognostic marker during cancer therapy, probably being indicative of high tumor mass. As the development of “Lab-on-the-chip” technology is advancing, simultaneous assessment of serum cytochrome c in combination with tissue and/or tumor-specific markers may one day allow precise definition of the tissue-specific cell death burden in vivo and thus allow for identification of “high-risk” patients. In such cases, an individualized, more aggressive therapy might be sought. Alternatively, apoptosis might be curative. Nevertheless, cancer type-specific studies that evaluate the prognostic value of serum cytochrome c during the therapy of different malignancies are necessary before the assay becomes the part of a standard diagnostic procedure in the clinic.

References


