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FULL PAPER

Apoptosis and inflammatory response in human astrocytes are induced by a transmissible cytotoxic agent of neurological origin

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SUMMARY

We demonstrated the presence of an *in vitro* transmissible cytotoxic agent (TCA) in the cerebrospinal fluid (CSF) of patients with different acute neurological diseases. The nature of this agent is still a matter of study since repeated attempts have failed to identify it as a conventional infectious agent. Here, we describe the mechanisms through which TCA affects human astrocytes, demonstrating:
a late apoptotic process, mediated by caspases 9 and 3 activation, involving the Bcl2-Bak-axis;
an early and late p38 MAPK activation;
an interference with the IL-8 and MCP-1 secretory response. These *in vitro* data provide initial evidence of TCA involvement as a pro-apoptotic and pro-inflammatory signal, directly affecting astrocytic behavior. The implications of these findings in certain neurological diseases will be discussed.

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INTRODUCTION

A transmissible cytotoxic agent (TCA), transferable *in vitro* to monkey epithelial kidney (VERO) cells, was initially isolated from a cerebrospinal fluid (CSF) sample of a patient with brain ischemia. Although the serial transmissibility of such cytotoxicity was in line with the presence of a virus, all the diagnostic protocols employed consistently failed to detect any trace of conventional viral agents. Intriguingly, we isolated several *in vitro* transmissible cytotoxicities, all named generically TCA, from CSF specimens from patients with different acute neurological disorders (Beretti *et al.*, 2006). Also in all those other cases, the search for cytopathic viruses consistently provided negative results.

Besides monkey VERO cells, several human cell types, including the astrocytoma cell line D54-MG (Portolani *et al.*, 2005), human primary lymphocytes (Portolani *et al.*, 2008) and primary human fibroblasts (Beretti *et al.*, 2011) were able to propagate TCA *in vitro*. Conversely, the RR4-murine microglial cell line, the prototype of brain macrophages, was unable to support TCA perpetuation *in vitro*. However, these immune cells were susceptible to TCA, responding with functional alterations including increased phagocytosis, impaired killing and enhanced production of MCP-1, TNF-α, nitric oxide (Beretti *et al.*, 2007). In line with these findings, Guerra *et al.* (2012) described in detail morphological changes and functional damage caused by TCA on various types of cells, including astrocytes, but the molecular events involved in cell response and eventually cell death remained poorly elucidated. Initial studies showed that five days after exposure to TCA, the cytotoxic effect on astrocytes peaked with 40% cell mortality: evidence of DNA fragmentation, increased intracellular calcium content and blebbing of the plasma membrane all hinted at an apoptotic process (Portolani *et al.*, 2008; Portolani *et al.*, 2005). Here, we focused on the bio-molecular mechanisms through which TCA affects human astrocytes. In particular, we investigated the nature of the cytotoxic damage, the activation of pathways involved in the event and the secretory response, if any.

Key words:
Cytotoxic factor, Misfolded proteins, Apoptosis, Chemokines/cytokines.

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MATERIALS AND METHODS

Cell cultures. D54-MG cells were grown in DMEM-F12 medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Euroclone, Milan, Italy). For experiments, D54-MG cells were seeded in 6-well (10^6cell/well) plates and at confluence (day 0), serum content was reduced to 2% and monolayers were exposed to mock or TCA preparations, according to previously described procedures (Portolani *et al.*, 2005). In parallel, cell stimulation with lipopolysaccharide (LPS) from *E. coli* serotype 0128:B12 (Sigma-Aldrich, St. Louis, MO, USA) was

also included as a positive control. Cell-free supernatants and cells were collected at 7 different time points (6, 18, 24, 48, 72, 96, 120 and 144 hours).

Cell treatment. Cells seeded in 6-well (10^6cell/well) plates were exposed to mock or TCA preparations as described above and treated or not with 10μM of the p38 inhibitor SB 203580 (Sigma-Aldrich, St. Louis, MO, USA).

Cytofluorimetric analyses. Apoptosis was quantitatively measured by cell cycle analysis with Propidium Iodide staining (Sigma-Aldrich, St. Louis, MO, USA) on fixed cells, as described in Guida *et al.* (2013) and Propidium Iodide/AnnexinV (Alexa Fluor 488, Life Technologies) co-staining assay (Maraldi *et al.* 2015). Data were obtained using flow cytometric analysis with FACScan within 1 h.

Western-blot. Whole cell lysates from D54-MG, treated with mock and TCA preparations at different time points were processed by western blot (WB) analysis, as previously described (Bertacchini *et al.*, 2013). Primary antibodies against active caspase 9, phosphorylated and total p38 MAPK (Cell Signalling, Danvers, MA, USA), caspase 3, Bcl-2 (Santa Cruz, CA, USA), and Bak (Epitomics, CA, USA) were employed. β-actin (Sigma-Aldrich, St. Louis, USA) was used as a control of protein loading.

Assessment of cytokine/chemokine levels by antibody microarrays. The levels of the cytokines IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-13, IFN-γ, TNF-α and chemokines IL-8 and MCP-1 were assessed in D54-MG cell-free supernatants obtained at 7 different time points (6, 18, 24, 48, 72, 96 and 144 hours) by means of the Quantibody® Human Inflammation Array 1 (Ray Biotech Inc., Norcross, GA, USA). In detail, the kit provides microarray slides, each spotted with 16 copies of a cytokine/chemokine antibody array. The slide comes with a 16-well removable gasket, which allows 16 samples to be processed on one slide. Every array consists of 6x8 matrices, made up with quadruplicates of each antibody and two positive controls. Prior to processing, the samples were diluted 1:10 in the appropriate diluent buffer provided in the kit and the assays were conducted according to a sandwich ELISA protocol, as per the manufacturer's instructions.
The fluorescent signal was read with a ScanArray Gx scanner (Perkin-Elmer, Cambridge, UK) and quantified with the ScanArray Express software (Perkin-Elmer).

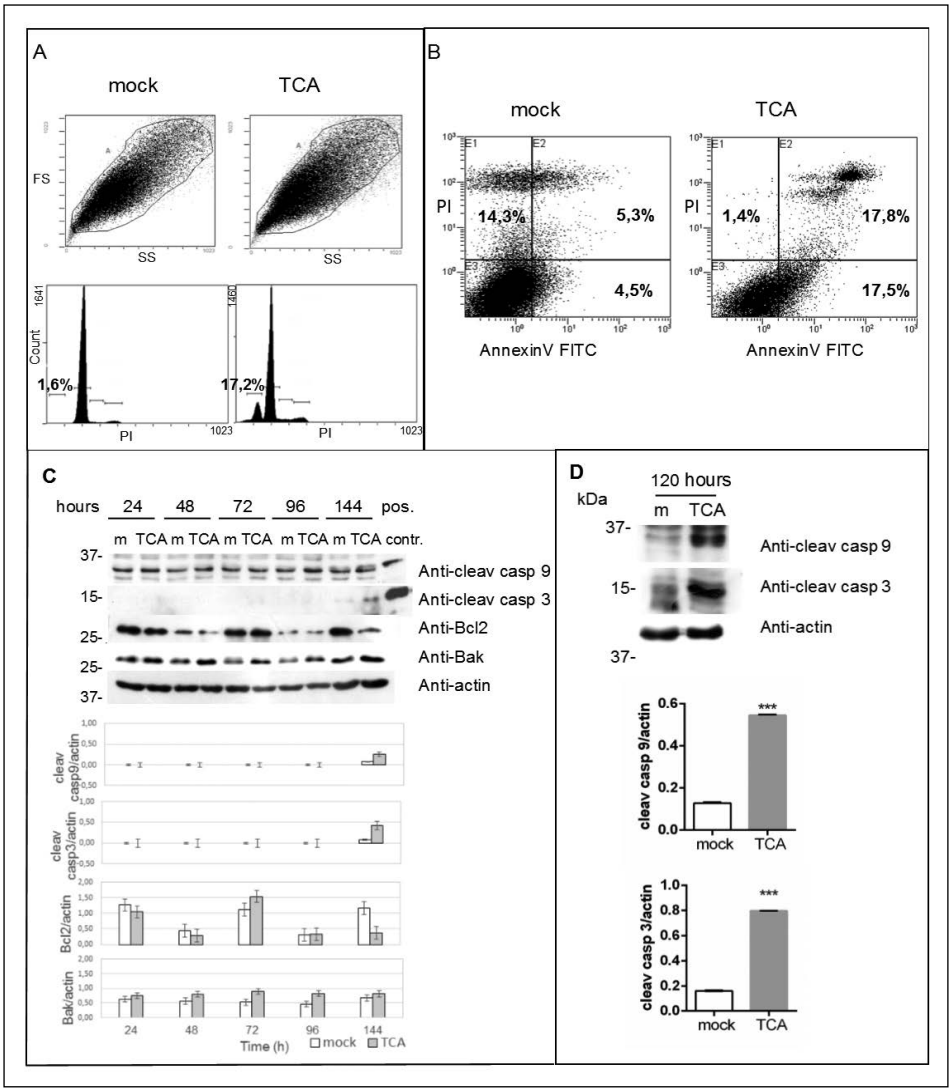

Figure 1 - *Apoptosis in D54-MG astrocytoma cells exposed or not to TCA. A), Day 5 cell cycle analysis with Propidium Iodide staining of TCA-treated cells and mock-treated control cells. Statistical analysis was performed using Student's t-test (TCA vs. controls); data are expressed as mean percentages (N=3). ***p≤0,001; B) Day 5 FACS analysis of Propidium Iodide/Annexin-V (Alexa Fluor 488, Life Technologies) co-staining of TCA-treated cells vs mock-treated control cells. Statistical analysis was performed using Student t-test (TCA vs. controls); data are expressed as mean percentages (N=3). *p<0,05; C) Western blot analysis of time course expression of caspases 3 and 9, Bak and Bcl-2. Relative levels were quantified by ImageJ density analysis; normalization was performed with actin; D) Western blot of active caspases 3 and 9 and actin in TCA-treated cells vs mock-treated control cells; relative levels of cleaved caspase 3 and 9 were quantified by ImageJ density analysis, normalization was performed with actin; Statistical analysis was performed using Student t-test (TCA vs. controls); data are expressed as means ± S.E.M. (N=3). ***p≤0.001.*

Each cytokine/chemokine was quantified by interpolating the fluorescent signals to the standard curve generated by processing some arrays with specific standards containing predetermined cytokine concentrations (pg/ml), as described in La Sala *et al.* (2012).

Statistical analysis. *In vitro* experiments were performed in triplicate. For quantitative comparisons, values were reported as mean ± SEM based on triplicate analysis for each sample. To test the significance of observed differences among the study groups, one-way ANOVA test with post-hoc Bonferroni correction or Student's t-test were applied. A P value <0.05 was considered statistically significant.

RESULTS

Cytofluorimetric analysis of apoptosis. In order to verify the occurrence of an apoptotic process and, at the same time, to assess its contribution to the cytotoxic damage, D54-MG astrocytes, challenged or not with TCA, were subjected to both Propidium Iodide staining (on fixed condition) and Propidium Iodide/AnnexinV co-staining assay (on fresh cells). As reported in *Figure 1A*, the rate of apoptosis, measured on fixed cells at 120 h, differed significantly between TCA-treated and mock-treated cells, namely 17.2%±0.9 and 1.6%±0.6 respectively (***, p<0,001; N=3). Similar results were obtained in parallel groups using

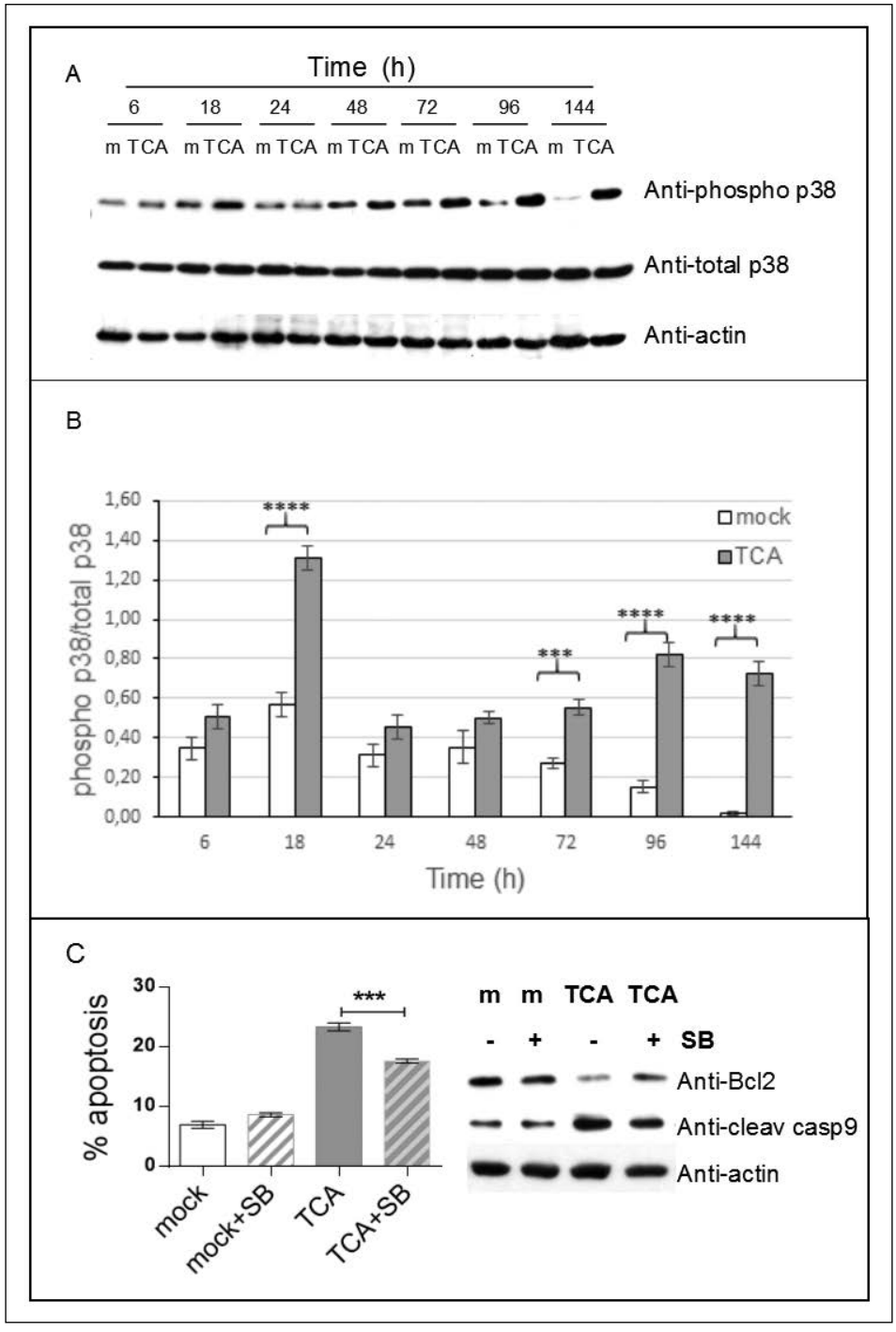

Figure 2 - *p38 MAPK expression and activation in D54-MG astrocytoma cells exposed or not to TCA. A) Time course of Western-blot analysis of phosphorylated and total p38 MAPK (Cell Signaling), actin used as loading control; B) Graphic presentation of density analysis as assessed by ImageJ. Values are means ±S.E.M. from three independent experiments. The statistical analysis was performed using Student t-test (TCA vs. controls) ***p<0.001, ****p<0.0001. C) Inhibition of p38 MAPK attenuates TCA-induced D54-MG apoptosis. Cells exposed or not to TCA were treated with 10μM SB203850. Apoptotic cells were examined by cytofluorimetric and Western blot analysis. All data are representative of three independent experiments. ***p<0.001 (TCA vs TCA+SB).*

Propidium Iodide/AnnexinV co-staining assay (*Figure 1B*). In particular, representative scatter plots show differences between mock- and TCA-treated cells in terms of:

a) viable cells (75.9%±5.5 vs 63.3%±1.6);
b) apoptotic cells (4.5%±1.4 vs 17.5%±3.5; p<0.05);
c) late apoptotic/secondary necrosis cells (5.3%±2.0 vs 17.8%±4.1;
d) necrotic cells (14.3%±4.4 vs 1.4%±0.8; p<0.05).

Similar analyses, carried out at days 4 and 6, never revealed significant differences between mock- and TCA-treated cells (data not shown).

Apoptotic process activation. To investigate the role of caspases in the TCA-induced apoptotic process, the activation of caspases 9 and 3 was assessed in TCA- and mock-treated cells by WB analysis. *Figure 1C* shows the expression of caspases 9 and 3 in a time course experiment. An increase in cleaved caspases occurred but only at the latest time point (i.e., 144 hours). Next, Bcl-2 and Bak expression was also analyzed. As depicted in *Figure 1C*, the pro-survival protein Bcl-2 decreased at 144 h, whereas the pro-apoptotic protein Bak increased from 48 hours up to 96 hours.

With the purpose of better addressing caspase involvement, an intermediate time point, namely 120 hours, was assessed by WB analysis. *Figure 1D* shows the activation of both enzymes; in particular, caspases 9 and 3 were cleaved significantly more in TCA-treated astrocytes than in mock controls, as shown by densitometric analysis.

p38 MAPK activation. The levels of p38 MAPK and the total/phosphorylated p38 MAPK ratio were investigated in D54-MG astrocytes, exposed or not to TCA. The amounts of total p38 MAPK were similar between mock- and TCA-treated samples (*Figure 2A*). Conversely, the total/phosphorylated p38 MAPK ratio in the TCA-treated specimens showed an early peak (at 18 hours) and a second peak that started at 72 hours and reached the highest values at 96 and 144 h. By contrast, mock-treated control cells showed a gradual reduction (*Figure 2B*). Furthermore, cell exposure to the p38 inhibitor SB 203580 (SB) caused a significant reduction of the apoptotic rate from 23.33± 0.67 to 17.67± 0.33 (TCA vs TCA+SB treated cells, respectively; *Figure 2C*).

Assessment of cytokine/chemokine levels. Experiments were performed to assess the levels of cytokine/chemokine produced by D54-MG cells in response to TCA. As shown in *Figure 3A*, supernatants from cells exposed to TCA displayed significantly higher levels of MCP-1 when compared to mock control cells (p<0.05) at 6, 18 and 24 h (*Figure 3A*). A similar response was observed in LPS-treated cells. As for IL-8 (*Figure 3B*), significantly higher levels were ob-

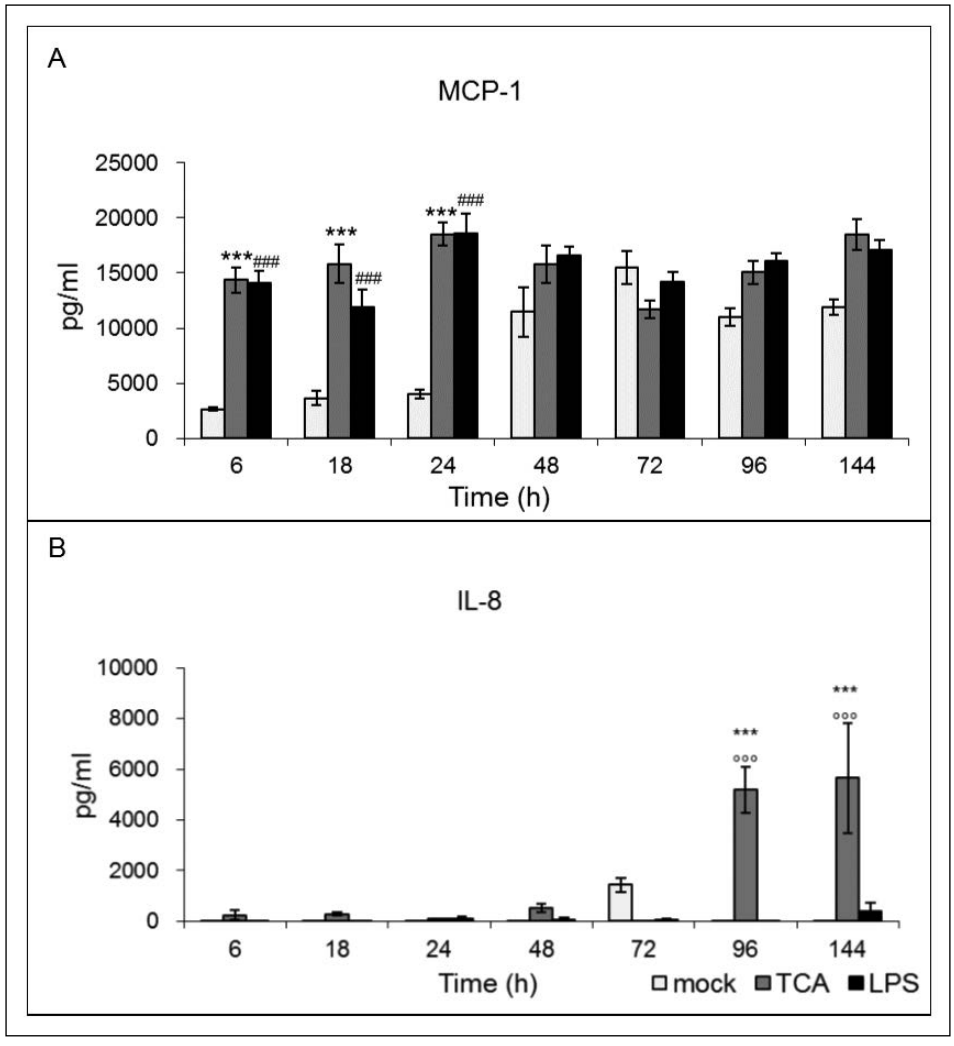

Figure 3 - *MCP-1 A) and IL-8 B) production in D54-MG astrocytoma cells exposed or not to TCA. Supernatant from cells exposed for the indicated times to TCA (grey columns) or mock (white columns), were assessed for cytokine content by microarray analysis. LPS (0.1 mg/ml for 1h, Sigma; black columns) was included as positive control. Data are expressed as means ±S.E.M., of quadruplicates. Statistical analysis was performed using one-way Anova analysis with Benferroni's multiple comparison test. ***p<0.05 (TCA vs control), ###p<0,05 (LPS vs control); °°°p<0.05 (TCA vs LPS).*

served in TCA-treated cells with respect to mock-controls, starting from 96 to 144 hours ($p<0.05$), whereas LPS failed to induce any appreciable IL-8 secretory response. Other cytokines were also tested; the only note of interest was an early trend towards an increase in the levels of the anti-inflammatory cytokines IL-10 and IL-13 in TCA-challenged cells (data not shown).

DISCUSSION

Here we show that the *in vitro* transmissible TCA of neurological origin affects human astrocytes, inducing apoptosis, p38 MAPK activation, IL-8 and MCP-1 production.
As detailed elsewhere (Beretti *et al.*, 2006), TCA has repeatedly been isolated from the CSF of patients with acute neurological disorders of different nature. The multiplicity of clinical pictures (brain ischemia, transverse myelitis, meningoencephalitis and multiple sclerosis at onset) rules out a direct etiological role for TCA in such diseases. Rather, our current hypothesis is that TCA is a cell product, locally generated at cerebral level in response to a variety of primary still unidentified insults. Concerning its biochemical nature, initial evidence suggests that TCA may be a prion-like protein as it shares several peculiarities with misfolded proteins, including cytotoxicity, resistance to proteolysis and a tendency to aggregation (Portolani *et al.*, 2005). Alternatively, TCA may be an antimicrobial peptide, since it retains functions such as inflammation induction and cytotoxicity, as well as extensively described antimicrobial peptides (Lehrer *et al.*, 1993; Magliani *et al.*, 2011).
As a step forward from our studies describing the cytotoxicity of TCA on different cell types of brain origin (Beretti *et al.*, 2006; Portolani *et al.*, 2005; Beretti *et al.*, 2007), this paper provides initial characterization of the bio-molecular events produced by TCA in human astrocytic cells. Here, we demonstrate that TCA induces an apoptotic process peaking at 120 h and occurring via caspase 3 and 9 activation, with Bcl-2 and Bak axis involvement. Nevertheless, the apoptosis occurs approximately in only half of the dead cells (18% apoptotic cells within 37% total dead cells). This suggests the co-existence of other mechanisms, that together with the caspase-dependent apoptosis, may contribute to the TCA-induced damage on astrocytes. As detailed by the cytofluorimetric data, apoptosis, necrosis and secondary necrosis are detectable in TCA-treated cells (*Figure 1B*) whereas mock cell death occurs only via a necrotic process, likely due to a natural aging of the culture (5 days old). In line with what has been described in virus-infected cells (Van den Berg *et al.*, 2013), we speculate that the programmed cell death observed only in TCA-treated cells is like a defense response, implemented by astrocytic cells to limit TCA perpetuation and its spread to other neighboring cells. It is worth noting that the D54-MG cells and other cell lines are successful tools for the *in vitro* propagation of TCA (Portolani *et al.*, 2005).
p38 MAPK phosphorylation is a crucial signaling step in a multiplicity of biological events such as inflammation, senescence and cell death (Zarubin *et al.*, 2005). The present model of astrocytes exposed to TCA showed an increase in phosphorylated p38 MAPK occurring with an early and late peak, and an inflammatory response, particularly in terms of enhanced production of MCP-1 and induction of IL-8. Interestingly, the early MCP-1 and the late IL-8 production appear to coincide with the dual peak of p38 MAPK activation. In our opinion, the inter-peak decrease may be interpreted as the result of a cell control system designed to limit the inflammation process triggered by the initial exposure to TCA. Nonetheless, a second peak of MAPK activation occurs and is likely associated with the fulfillment of the apoptotic event. In line with this hypothesis, we provide evidence that inhibition of p38 activation leads to a significant reduction of the apoptotic process, as also supported by WB data showing a decrease in Bcl-2 and cleaved caspase 9. It is noteworthy that the TCA pro-inflammatory signal is more efficacious than that mediated by LPS, at least in terms of IL-8 response by astrocytes. This implies that, once produced, TCA may have a potent role in affecting neighboring cell behavior.
Overall, the present findings suggest that astrocytic cell response to TCA is a complex process, involving temporally distinct phases that gradually allow signal transduction, secretory response and programmed cell death. Notoriously, astrocytes play a key role in driving/amplifying local inflammatory processes during acute neurological disorders (Fouillet *et al.*, 2012; Amor *et al.*, 2014; Viviani *et al.*, 2014). Moreover, recent data suggest that deviations from MAPK signaling pathways may have implications in brain ischemia and neurodegenerative diseases (Bachstetter *et al.*, 2014) where the role of inflammation also appears to be crucial. If our *in vitro* data prove to have an *in vivo* counterpart, we may assume the involvement of TCA in neurological diseases through astrocytes that not only efficaciously propagate such a cytotoxic agent, but also respond to it with activation, inflammatory response and programmed cell death.

Abbreviations

TCA, transmissible cytotoxic agent; CSF, cerebrospinal fluid; LPS, lipopolysaccharide.

Funding

None

Conflict of Interest

The authors declare that they have no conflict of interest.

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