Summary. The aim of the present work was to investigate the occurrence and immunological characteristics of chronic carotid glomitis in opiate addicts. Carotid bodies were sampled at autopsy from 50 subjects who died of heroin intoxication (mean age 28 years), and from 16 young (24 years) and 10 older subjects (66 years) who died of trauma. Sections were stained with haematoxylin-eosin and azan-Mallory, and immunohistochemistry was carried out with anti-CD45, -CD3, -CD8, -CD4, -CD20, -CD68, -CD56. Inflammatory aggregates were not observed in young cases, but were found in 21/50 (42%) opiate cases and in 4/10 (40%) older cases. Infiltrates were mainly located in subcapsular and interlobular positions, and were also found around nerve fibres. Inflammatory aggregates were mainly composed of T suppressor/cytotoxic lymphocytes (50-80%). Monocytic/macrophagic cells and B lymphocytes comprised about 10% and 5-20% of inflammatory cells, respectively. T helper lymphocytes were fewer and only rare Natural Killer cells were found. Chronic carotid glomitis must be included among the autopsy findings of opiate addiction, and may be ascribed to inflammatory reactions to exogenous immunogens or to responses to drug-induced degenerative changes of carotid body components.

Key words: Carotid body, chronic glomitis, Heroin, addiction, Nervous system, Autonomic

Introduction

The carotid body is a small ovoid tissue mass situated at the carotid bifurcation, which plays a role as arterial chemoreceptor, inducing increases in ventilatory volume and frequency in response to hypoxia, hypercapnia, or reduction of blood pH. The lobules of the carotid body, separated by connective septa, are organised in clusters of two different cell types, i.e., type I (or chief) cells, in turn separated into light, dark and pyknotic, and type II (or sustentacular) cells (Verna, 1979; Smith et al., 1982; Pallot et al., 1986; Pallot, 1987). Type I cells store neurotransmitters/neuromodulators (Nurse, 2005; Porzionato et al., 2006) and are considered the chemoreceptor elements of the carotid body. Type II cells show astrocytic markers and play a supportive role (Pallot et al., 1986; Pallot, 1987), although it has recently been observed that these cells exposed to prolonged hypoxia may behave as stem cells precursor for type I cells (Pardal et al., 2007). In the carotid body, chemosensory impulses are mainly conveyed through glosso-pharyngeal afferent fibers arising from the petrosal ganglion (Pallot, 1987). In addition, the carotid body shows sensory innervation from the jugular and nodose ganglia of the vagus, and also receives a complex sympathetic and parasympathetic innervation, which modulates chemosensory activity through actions on the vasculature and type I cells (reviewed in Ichikawa, 2002).

Morphological and functional changes have been reported as a consequence of chronic hypoxia (Wang and Bisgard, 2002; Kusakabe et al., 2002, 2004, 2005; Prabhakar and Jacono, 2005; Lam et al., 2008). Moreover, carotid body alterations have been reported in ageing (Hurst et al., 1985; Heath and Smith, 1992; Di Giulio et al., 2003; Pokorski et al., 2004) and in various pathologies, such as respiratory diseases (Heath et al., 1990; Heath and Smith, 1992; Bencini and Pulera, 1991; Heath, 1991), cardiac malformations (Smith et al., 1986) and sudden infant death syndrome (Gauda et al., 2007; Porzionato et al., 2008a). In ageing, carotid body changes include increase of connective tissue, reduction of carotid body parenchyma and proliferation of type II cells (Hurst et al., 1985; Heath and Smith, 1992; Porzionato et al., 2005). In the literature, sparse and diffuse inflammatory infiltrates within the carotid body,
although more frequent in ageing, have also been reported in subjects younger than 50, but chronic carotid glomitis, a pathological condition defined by the presence of aggregates of lymphocytes throughout the carotid body, has only been described over the age of 50. It has been considered a response to degenerative changes occurring with age; relationships with specific diseases have not yet been proposed (Hurst et al., 1985; Khan et al., 1989; Heath and Khan, 1989; Khan and Heath, 1990). In the carotid body of heroin addicts, specific histopathological changes have also been identified, i.e., increase in connective tissue and type II cells, and decrease in chief and light cells (Porzionato et al., 2005). In particular, intra- and interlobular fibrosis and type II cell hyperplasia have been interpreted as signs of early tissue ageing. However, the presence of inflammatory infiltrates and the occurrence of chronic carotid glomitis in heroin addiction has not been previously investigated.

The present study was designed to investigate the occurrence of chronic carotid glomitis in opiate addicts who died of heroin intoxication, control groups being healthy young and old subjects. Immunological characterisation of inflammatory aggregates within the carotid body was also performed.

Materials and methods

Materials

Carotid bodies were obtained at autopsy from 50 subjects who died of heroin/morphine intoxication (38 male, 12 female; mean age (± SD) 28±2.6 years). Twenty-nine subjects had also been included in a previous work considering other histopathologic changes of the carotid body (Porzionato et al., 2005). In all cases, there was a clinical history of at least 3 years of heroin addiction. Controls were divided into two groups: 16 young (10 male, six female; mean age 24±3.6 years) and 10 older subjects (7 male, three female; mean age 66±3.8 years), who had died shortly after traumas. All subjects were clinically negative for infective, chronic pulmonary or cardiovascular diseases. Autopsies from patients with cardiac hypertrophy or myocardial infarction were excluded from this study. Autopsies were performed between 18 and 78 hours after death. Specimens were taken of both carotid bifurcations, including 2 cm of the common carotid and 2 cm of the internal and external carotid arteries. In each case, multiple samples from the major organs were also performed for histological examination.

Toxicological examination

Confirmation of heroin intoxication and a search for other drugs of abuse were performed by toxicological immunochemical screening (enzyme multiplied immunoassay technique) and confirmatory chromatographic techniques (high-performance liquid-chromatography and gas-chromatography, coupled with mass spectrometry in selective ion monitoring mode) of urine and central venous blood samples.

Histological and immunohistochemical techniques

Specimens were fixed in neutral 10% formalin and embedded in paraffin wax. For each case, histological examination was carried out on five longitudinal sections, 5 µm thick, at 50 µm distance from each other and stained with haematoxylin-eosin and azan-Mallory. In accordance with the literature, chronic carotid glomitis was specifically diagnosed if prominent aggregates of lymphocytes were found in the carotid body.

Immunological characterisation of the inflammatory infiltrates within the carotid body was carried out with the following antibodies diluted in Phosphate-Buffered Saline (PBS): anti-CD45 (Monoclonal Mouse Anti-Human CD45, Leucocyte Common Antigen, Clones 2B11 + PD7/26, M 0701, DAKO, Milan, Italy) 1:400; anti-CD68 (Monoclonal Mouse Anti-Human CD68, Clone KP1, M 0814, DAKO) 1:200; anti-CD3 (Polyclonal Rabbit Anti-Human CD3, A 0452, DAKO) 1:50; anti-CD20 (Monoclonal Mouse Anti-Human CD20cy, Clone L26, M 0755, DAKO) 1:200; anti-CD8 (Monoclonal Mouse Anti-Human CD8, Clone C8/144B, M 7103, DAKO) 1:100; anti-CD4 (Monoclonal Mouse Anti-Human CD4, Clone 1F6, NCL-CD4-1F6, Novocastra Laboratories, Newcastle, UK) diluted 1:100; and anti-CD56 (Monoclonal Mouse Anti-CD56 (NCAM), NCL-CD56-1B6, Novocastra) diluted 1:100.

Sections were hydrated gradually through decreasing concentrations of ethanol and then washed in deionised H2O. They were incubated in 1% hydrogen peroxide in H2O. They were incubated in 1% hydrogen peroxide in 90° C for 20 min; for anti-CD68 immunohistochemistry, unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 90°C for 20 min; for anti-CD68 immunohistochemistry, sections were incubated with 0.01 M PBS containing 0.1% Triton® X-100 for 10 min; for anti-CD4 immunohistochemistry, unmasking was performed with 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, at 90°C for 20 min. Unmasking was not performed in the other immunohistochemical protocols. Sections were then incubated for 30 min in blocking serum (0.04% bovine serum albumin (A2153, Sigma-Aldrich, Milan, Italy) and 0.5% normal goat serum (X0907, Dako Corporation, Carpinteria, CA, USA) to eliminate unspecific binding, and then incubated for 1 hour at room temperature with the above primary antibodies. Primary antibody binding was revealed by incubation with anti-rabbit/mouse serum diluted 1:100 in blocking serum for 30 min at room temperature (DAKO® EnVision + TM Peroxidase, Rabbit/Mouse, Dako Corporation, Glostrup, Denmark) and developed in 3,3'-diaminobenzidine for 3 min at room temperature. Lastly,
Sections were counterstained with haematoxylin. Sections incubated without primary antibodies showed no immunoreactivity, confirming the specificity of the immunostaining.

Statistical analysis

For the presence of chronic carotid glomitis, groups were compared with Kruskal-Wallis test and Dunn’s multiple comparison test. For each substance, toxicological values were compared with Mann-Whitney test between opiate-related deaths with and without chronic carotid glomitis. P-value of 0.05 was considered to be significant. Statistical calculations were performed with Statigraphic 4.0 software (STSC Inc., Rockville, MD, USA).

Results

Pathological and toxicological findings are given in Table 1. In 44 cases, a haemorrhagic pulmonary edema was found, in three cases massive occlusion of respiratory airways by gastric contents, and in three cases hypoxic/ischaemic brain lesions. In all these cases, morphine was detected in serum (free morphine range: 0.1-31.1 µg/ml) and in 29 cases also in urine (free morphine range: 0.1-103.2 µg/ml). In 20 cases, alcohol was detected in serum (range: 30-222 mg/100ml) and in 14 cases also in urine (range: 10-276 mg/100ml). In one case, methadone was found in serum (3.4 µg/ml). In 8 cases, benzodiazepines were detected in both serum (range: 0.7-1.3 mg/ml) and urine (range: 0.3-9.7 mg/ml). In 2 cases, tetrahydrocannabinol was detected in serum (range: 30-222 mg/100ml) and urine (range: 0.3-8.7 mg/ml).

Table 1. Toxicological findings of opiate cases, subdivided on the basis of presence or absence of chronic carotid glomitis.

<table>
<thead>
<tr>
<th>Presence of Chronic Carotid Glomitis (21 cases)</th>
<th>Morphine-S (µg/ml)</th>
<th>Morphine-U (µg/ml)</th>
<th>Alcohol-S (mg/100ml)</th>
<th>Alcohol-U (mg/100ml)</th>
<th>BDZ-S (mg/ml)</th>
<th>BDZ-U (mg/ml)</th>
<th>THC-S (ng/ml)</th>
<th>THC-U (ng/ml)</th>
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<tr>
<td>n (range)</td>
<td>Mean±SD</td>
<td>n (range)</td>
<td>Mean±SD</td>
<td>n (range)</td>
<td>Mean±SD</td>
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<td>Mean±SD</td>
<td>n (range)</td>
</tr>
<tr>
<td>Presence of Chronic Carotid Glomitis (21 cases)</td>
<td>21 (0.1-5.5)</td>
<td>2.0±1.5</td>
<td>16 (0.1-7.1)</td>
<td>1.8±2.0</td>
<td>8 (30-200)</td>
<td>100±68.2</td>
<td>7 (10-264)</td>
<td>119.3±103</td>
</tr>
<tr>
<td>Absence of Chronic Carotid Glomitis (29 cases)</td>
<td>29 (0.1-31.1)</td>
<td>3.9±5.7</td>
<td>13 (0.1-103.2)</td>
<td>14.2±28.7</td>
<td>12 (30-222)</td>
<td>116.6±87.0</td>
<td>7 (41-276)</td>
<td>119±97.5</td>
</tr>
<tr>
<td>S: serum; U: urine; BDZ: benzodiazepine; THC: tetrahydrocannabinol; n: number of positive cases; SD: Standard Deviation.</td>
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a decrease in chief cells (Fig. 4A,B).

**Discussion**

Infections are a leading cause of morbidity among heroin addicts (Scheidegger and Zimmerli, 1989) and chronic inflammatory changes have been reported in various organs and tissues, such as skin (chronic inflammation of injection marks) (Kringsholm and Christoffersen, 1989), kidney (mono-lymphocytic membranoproliferative glomerulonephritis, focal segmental sclerosing glomerulonephritis, granulomatous glomerulonephritis, renal interstitial fibrosis) (Eknoyan et al., 1973; Rao et al., 1974; Cunningham et al., 1980; Singhal et al., 1997; Segal et al., 1998; Dettmeyer et al., 1998, 2000), lung (granulomatous reactions) (Kringsholm and Christoffersen, 1987; Wolff and O'Donnell, 2004), heart (endocarditis, lymphocytic infiltrations of conduction tissue) (Reisberg, 1979; Kringsholm and Christoffersen, 1987) and brain (perivascular mono-lymphocytic infiltrates, microglial proliferation) (Makrigeorgi-Butera et al., 1996; Davies et al., 1997; Tomlinson et al., 1999). Perivascular infiltrates mainly composed of CD8+ T cells have been reported in the brain of HIV-seronegative or -seropositive drug addicts (Makrigeorgi-Butera et al., 1996; Tomlinson et al., 1999), although some authors did not find a statistically significant difference between HIV-seronegative drug users and non-drug users (Tomlinson et al., 1999). Instead, the average number of CD68+ microglia has been found to be significantly increased in HIV-seronegative drug users as compared to non-drug.
Fig. 2. Sections of carotid body of opiate-related death immunohistochemically stained with anti-CD45 (A-B), -CD3 (C), -CD68 (D), -CD20 (E-F), showing an inflammatory aggregate mainly composed of CD3+ T lymphocytes (C) and CD68+ monocytic-macrophagic cells (D). CD20+ B lymphocytes are fewer (E). F, an inflammatory infiltrate with a higher percentage of CD20+ lymphocytes. Scale bars: A, 200 µm; B, 100 µm; C-F, 50 µm.
users (Davies et al., 1997; Tomlinson et al., 1999). To the best of our knowledge, this is the first study reporting chronic carotid glomitis in heroin addicts. In the literature, focal aggregates of lymphocytes had been reported only in subjects over the age of 50 (Hurst et al., 1985; Khan et al., 1989, 1990; Heath and Khan, 1989; Khan and Heath, 1990). In our study, we confirmed the possible occurrence of inflammatory infiltrates in older but not in younger controls, age-matched with opiate-related deaths. Thus, chronic carotid glomitis may be included in the series of pathological findings observed at autopsies of opiate addicts.

The immunological characteristics of inflammatory infiltrates within carotid body tissue, showing a prevalence of T cells (CD3+), were in keeping with literature data on chronic carotid glomitis in the ageing population (Khan et al., 1989). The great majority of T cells were T suppressor/cytotoxic lymphocytes (CD8+), comprising 50-80% of inflammatory cells. T helper lymphocytes (CD4+) were poorly represented and Natural Killer cells (CD56+) were rarely found. Conversely, monocytic/macrophagic cells (CD68+) comprised about 10% of inflammatory cells in all cases of chronic carotid glomitis. The immunological characteristics of inflammatory infiltrates of the carotid body in heroin addiction fit the inflammatory reactions reported in the other tissues of opiate-related deaths, with both lymphocytic and monocytic/macrophagic components.

Infectious diseases in drug users have been ascribed to unsterile injection techniques and to immunosuppression caused by chronic opiate abuse (Karch, 1996; Büttner et al., 2000; Wolff and O'Donnell, 2004). Immunological reactions caused by heroin itself or

Fig. 3. Sections of carotid bodies of opiate-related deaths immunohistochemically stained with anti-CD8 (A-B), -CD4 (C) and -CD56 (D), showing infiltrates with massive CD8+ T suppressor/cytotoxic lymphocytic component (A-B), fewer CD4+ T helper lymphocytes (C), and an occasional CD56+ Natural Killer cell (D, arrow). Note CD56 expression also in glomus cells. Scale bars: A-B, 100 µm; C-D, 50 µm.
Chronic carotid glomitis in addiction

Fig. 4. Sections of carotid body showing an increase in interlobular connective tissue in an opiate-related death with chronic carotid glomitis (B; arrow: inflammatory aggregate) as compared with a young case (A) (azan-Mallory). C-F. Sections of carotid body, showing relationships between inflammatory aggregates and connective tissue (H.E.: C, E; azan-Mallory: D, F). Scale bars: A, B, 400 µm; C, D, 200 µm; E, F, 50 µm.
injected substances have also been proposed in the pathogenesis of heroin-associated glomerulonephritis or other inflammatory reactions (Dettmeyer et al., 1998). Chronic carotid glomitis in heroin addiction may also be ascribed to inflammatory reactions to immunogens, such as infective agents, heroin itself, other drugs or adulterants. The carotid body is the structure in the body with the highest blood flow (about 1400 or 2000 ml/100 g/min in the cat, depending on the technique for determination of tissue blood flow) (Daly et al., 1954; Barnett et al., 1988), and this may help to concentrate immunogens and inflammatory cells. The occurrence in the carotid body of heroin addicts of histopathologic changes similar to ageing, i.e., chronic carotid glomitis, sustentacular cell hyperplasia, fibrosis and atrophy, suggests a drug-induced acceleration of a natural degenerative process. In particular, as already suggested in ageing (Khan et al., 1989), chronic carotid glomitis may be a response to degenerative changes of the carotid body components. Moreover, it has been reported in the literature that heroin topically applied to Mitchell's central chemoreceptor area induces ventilatory depression, whereas heroin intravenous administration is still effective after bilateral section of vagus and carotid sinus nerves, thus suggesting ventilatory depression mainly by acting at central sites (Taveira et al., 1983). In response to central hypoventilation produced by heroin, arterial chemoreceptors sustain a high discharge rate of chemosensory discharges. Thus, chronic carotid glomitis may also be the consequence of the repeated hypoxic challenges suffered by heroin users. Recurrent episodes of hypoxia may occur under the effects of opiates or other respiratory depressants (alcohol, benzodiazepines), stimulating lympho-monocytic proliferation within the carotid body.

Otherwise, inflammatory cytokines may act directly on glomus cells and nerve fibres, modifying the chemosensitive function of the carotid body. IL-1 and IL-6 receptors have been identified in type I cells in the rat carotid body (Wang et al., 2002, 2006). It has been hypothesised that these cytokines alter the excitability of glomus cells and modify the electric activity of chemosensory nerve fibres, or modulate survival, proliferation and differentiation of glomus cells (Wang et al., 2002, 2006; Porzionato et al., 2008b).

In conclusion, in the complex clinicopathological entity which is opiate addiction, we may hypothesise different pathogenetic mechanisms for both degenerative changes and inflammatory reactions. It has been stressed that, in heroin addiction, the increases in connective tissue and type II cells and the decrease in chief and light cells may play a role in the fatal cardiorespiratory derangement (Porzionato et al., 2005). Inflammatory infiltrates in the carotid body may also contribute to chemosensitive impairment in heroin addicts. Due to the possible occurrence of chronic carotid glomitis and other pathological findings in the carotid body (an increase in connective tissue and type II cells, a decrease in chief and light cells) (Porzionato et al., 2005), sampling and analysis of this structure should be recommended in opiate addiction.

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References


Chronic carotid glomitis in addiction


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