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Pathological Changes in Masked Palm Civets Experimentally Infected by Severe Acute Respiratory Syndrome (SARS) Coronavirus

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Summary

Masked palm civets are highly susceptible to infection with the severe acute respiratory syndrome coronavirus (SARS-CoV). Infected animals become less aggressive and develop pyrexia, lethargy and diarrhoea. The present study describes the spectrum of histopathological changes in the lung, spleen, lymph node, liver, small intestine, kidney and cerebrum of civets infected experimentally with SARS-CoV. In-situ hybridization (ISH) with probes specific for the RNA polymerase gene demonstrated viral RNA in the lung, small intestine and cerebrum only. In-situ labelling was employed in order to demonstrate cellular apoptosis in the cerebrum, but there was no evidence of apoptosis within the myocardium. These results indicate that SARS-CoV causes multi-organ pathology in civets, similar to that observed in human SARS patients. These parallels suggest that civets may be used as an animal model of this infection to gain insight into the pathogenesis of SARS and for evaluation of candidate vaccines and antiviral drugs.

Keywords: apoptosis; civet; in-situ hybridization; SARS coronavirus

Introduction

Severe acute respiratory syndrome (SARS) first emerged in Guangdong Province in the People's Republic of China in November 2002. The aetiological agent of this syndrome was a newly emerged and previously unrecognized coronavirus, now known as SARS coronavirus (SARS-CoV) (Kuiken *et al.*, 2003; Ksiazek *et al.*, 2003). SARS is an acute pulmonary syndrome characterized by atypical pneumonia, progressive respiratory failure and death in up to 10% of infected individuals (Poon *et al.*, 2004). Although the SARS epidemic has subsided, many authorities, including the World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC), have warned of the possible re-emergence of this highly infectious disease. It is therefore imperative that effective measures to prevent and treat the disease are developed and evaluated. To achieve this goal, animal models will play an essential role in studying the pathogenesis of SARS-CoV infection and in developing effective vaccines and therapeutics.

A wide range of animal species has been confirmed to be susceptible to experimental infection with SARS-CoV, including rodents (mice and hamsters), carnivores (ferrets and cats) and non-human primates (Wang *et al.*, 2006). Adult mice infected with SARS-CoV *via* the respiratory tract show no clinical signs of disease and only mild respiratory tract inflammation (Subbarao *et al.*, 2004). Aged mice, hamsters and ferrets do show signs of clinical disease such as weight loss and ruffled fur but do not develop lung pathology (Martina *et al.*, 2003; Roberts *et al.*, 2005a,b). Two groups of investigators have studied SARS-CoV

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infection in African green monkeys and common marmosets (Kuiken *et al.*, 2003; McAuliffe *et al.*, 2004; Greenough *et al.*, 2005). Others have evaluated cynomolgus monkeys and rhesus macaques as potential models, but clinical disease is inconsistently induced in these latter species (Kuiken *et al.*, 2003; McAuliffe *et al.*, 2004; Rowe *et al.*, 2004).

Our laboratory has evaluated the suitability of guinea-pigs, hamsters, albino hamsters, chickens and rats as experimental models following inoculation of SARS-CoV strain BJ01. No clinical signs or tissue histopathological changes were observed in any of these animals post-infection. By contrast, all cynomolgus monkeys and rhesus macaques inoculated in this manner developed interstitial pneumonia of variable severity but no other tissue changes (Liu *et al.*, 2004). This pulmonary pathology was similar in nature to that seen in SARS patients, but the lesions were less severe than in infected humans. Therefore, there remains a requirement for an animal model of human SARS that closer approximates the tissue pathology that occurs in the human disease.

In October 2003 it was reported that SARS-CoV-like viruses had been isolated from masked palm civets from a live animal market in Guangdong, China (Guan *et al.*, 2003). This report prompted us to examine the feasibility of using civets as a better animal model for SARS. In a preliminary infection study, two groups of civets were inoculated with two different strains of SARS-CoV. Strain GZ01 was isolated during the early stages of the SARS epidemic and strain BJ01 was isolated during the middle phase of the epidemic (Wu *et al.*, 2005). Both strains were shown capable of infecting civets and inducing clinical signs including pyrexia, lethargy and loss of aggression (Wu *et al.*, 2005). The aim of the present study was to extend these observations by characterizing the tissue pathology in the infected civets and determining the distribution of viral RNA in tissues from those animals. In addition, analysis of cellular apoptosis in selected tissues was also conducted in an attempt to understand the pathogenesis of this viral infection in civets and to further determine the suitability of this species to model the human disease.

Materials and Methods

Virus, Animals and Inoculation

The animals and groups used in this study correspond exactly to those described in a previous publication (Wu *et al.*, 2005). SARS-CoV isolates were propagated in Vero E6 cells for two additional passages to generate virus stocks with titres of 1×10^6 50% tissue culture infective doses ($TCID_{50}$)/ml. Ten one-year-old masked palm civets were housed in individual biosafety isolators and were divided into two groups ($n = 5$ per group). Animals in groups A and B were inoculated with 3 ml of virus solution containing 3×10^6 $TCID_{50}$ of BJ01 and GZ01 isolates, respectively, with 2 ml instilled into the trachea and 1 ml given intranasally. A control civet was mock-infected in an identical fashion with 3 ml of Vero E6 cell culture supernatant. All work with infectious virus was performed inside a biosafety cabinet, in an approved animal biosafety level 3 laboratory. Serology and polymerase chain reaction (PCR) analysis were conducted to confirm that the civets had not been previously exposed to SARS-CoV. Animal experiments were conducted in accordance with the animal ethics guidelines and approved protocols issued by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Histopathology

One animal from each group was sacrificed at 3, 13, 23, 34 and 35 days post-infection (dpi), and lung, spleen, lymph node, small intestine, kidney, trachea, cerebrum, pancreas, sex glands, stomach and heart were collected from each animal. These samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections taken from these blocked samples were stained by haematoxylin and eosin (HE).

In-situ Hybridization (ISH)

Two 3-digoxigenin-labelled oligonucleotide probes (Probe 1: 5′-acc ctg cta aag cat ata agg att acc tag-3′; Probe 2: 5′-CAA TGG CTG ATT TAG TCT ATG CTC TAC GTC-3′), which target the RNA polymerase gene of SARS-CoV, were purchased from Invitrogen (Carlsbad, California, USA). ISH was performed on the full range of tissues collected from each animal with ready-to-use reagents purchased from Boster Biological Technology Co. Ltd. (Wuhan, China) following the manufacturer's instructions. Briefly, tissue sections were de-waxed in xylene and rehydrated in gradient ethanol. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2. The tissues were digested with 3 mg/ml pepsin in 0.14 M citric acid at 37°C for 20 min and incubated at 37°C for 4 h with pre-hybridization buffer containing 35% deionized formamide, 5 × standard saline citrate, 2% blocking reagent, 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulphate and 100 ng/ml salmon sperm DNA. The tissue sections were hybridized with digoxigenin-labelled