Research Report

In vivo imaging of apoptosis in patients with acute stroke: Correlation with blood–brain barrier permeability

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ABSTRACT

Background: We wished to determine the ability of radiolabeled annexin V to concentrate at sites of ischemic injury in patients with acute cerebral stroke. Secondly, we sought to correlate annexin V imaging in these patients with the degree of blood–brain barrier (BBB) breakdown. Methods: Twelve patients with acute stroke had a complete neurological examination, including the National Institutes of Health (NIH) stroke scale and the Glasgow Coma Score (GCS). A non-contrast CT scan was performed on all patients. A SPECT of the brain was obtained 2 h after injection of annexin V. The integrity of the BBB was evaluated in seven patients using Tc-99m-DTPA brain SPECT. Results: All patients had an infarct in the MCA territory. Eight patients had abnormal increased annexin V activity, which was more common in patients with cortical strokes (P = 0.01). The concentration of annexin had no correlation to the volume of stroke, but it was significantly and inversely related to the GCS on admission (r = −0.7, P = 0.02). Foci of apoptosis were noted contralateral to the affected hemisphere as well. All seven patients who underwent DTPA SPECT showed breakdown of the BBB. DTPA uptake was significantly and positively associated with NIH score (r = 0.80, P = 0.01) and inversely associated with GCS (r = −0.89, P = −0.03). Conclusion: This study shows that it is possible to identify in vivo regions of ischemic neuronal injury using radiolabeled annexin V in patients with acute stroke. Annexin imaging can play a major role in the selection of therapy in the initial period following stroke in adults.

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1. Introduction

The use of technetium-99m hydrazine nicotinamide labeled annexin V (99mTc-HYNIC-annexin V), an in vivo imaging marker of apoptosis, has been reported in several clinical investigations of acute heart transplant rejection, myocardial ischemia, and tumor treatment (Belhocine et al., 2002; Haas et al., 2004; Kartachova et al., 2004; Narula et al., 2001; Thimister et al., 2003). However, there have been no published reports on the clinical use of 99mTc-HYNIC-annexin V for the imaging of patients suffering from cerebral artery stroke or other neurologic diseases such as multiple sclerosis, Alzheimer’s, ALS, and the autoimmune cerebral vasculitides in which apoptosis may play a significant role (Du et al., 1996; Hara et al., 1997; Thompson, 1995; Vexler et al., 1997).
In one experimental model, \(^{99}\text{Tc}\)-HYNIC-annexin V imaging was able to detect neuronal apoptosis within 2 h after reversal of global hypoxia (D’Arceuil et al., 2000). Abnormally increased uptake of annexin V occurred despite the presence of an intact blood–brain barrier (BBB) as assessed by co-injection of In-111 DTPA and dual energy scintigraphy as well as concurrent Gd-DTPA bolus tracking including delayed post-Gd-DTPA MR imaging of the brain and cerebellum. In a second model, remarkable focal radiolabeled annexin uptake was observed as soon as 2 h after reperfusion injury, well before any evidence of BBB breakdown as confirmed by concurrent Gd-DTPA bolus perfusion and delayed post-Gd-DTPA MR scanning (Mari et al., 2004). In both models, \(^{99}\text{Tc}\)-HYNIC-annexin V was co-injected with equimolar amounts of fluorescent labeled annexin V. Surprisingly, fluorescent annexin V was able to pass the intact BBB and localize within the cytoplasm of ischemic neurons despite its relatively high molecular weight of 35 kDa. These results suggest the existence of an active annexin V uptake mechanism by which this protein can cross the BBB and the cell membrane of ischemic or “physiologically stressed” neurons.

Other studies on animal models have shown that reduced blood flow to the brain can alter the BBB permeability and regulatory transport functions (Belhocine et al., 2002; Blankenberg et al., 1998; Vriens et al., 1998). We have shown in a human study that \(^{99}\text{Tc}\)-DTPA brain SPECT is the technique of choice for the assessment of BBB disruption. Combined with quantitative analysis in patients with acute stroke, SPECT imaging of the BBB is significantly related to clinical outcome (Lorberboym et al., 2003).

Considering that stroke is the third most common cause of death and a major cause of long-term disability (Peters et al., 1988), imaging studies have an important role in assessing the location and extent of the stroke, in determining tissue viability, in patient prognosis and in clinical management. Understanding the pattern and distribution of apoptosis in stroke may elucidate the mechanisms of neuronal injury and repair, which may lead to new therapies that may prevent neuronal loss.

In this current investigation, we wished to determine if \(^{99}\text{Tc}\)-HYNIC-annexin V SPECT imaging could detect sites of ischemic injury as identified on CT scanning on admission of patients suffering from an acute stroke. Secondly, we wished to correlate annexin V uptake with the location and degree of disruption of the blood–brain barrier (BBB) as assessed by follow up SPECT with \(^{99}\text{Tc}\)-DTPA performed 1 day after annexin V imaging.

2. Results

2.1. Normal distribution of annexin V

At 2 h post-injection of Tc-99m-annexin V in all five control patients, activity was noted in the calvarium, venous sinuses and choroids plexus, with minimal blood-pool activity. No parenchymal uptake of annexin V was observed within the cerebrum or posterior fossa in control patients.

2.2. The volume of stroke

Abnormal annexin uptake had no correlation to the volume of stroke as at CT. The volume of stroke was significantly related to the NIH and GCS scores (\(P = 0.01\)).

2.3. Annexin V imaging in patients with acute stroke

Eight patients had abnormal increased uptake of annexin V corresponding to the pathological infarct regions identified by CT (Fig. 1). The mean AI was 4.7 ± 4.7 (range 1.6–15.6, Table 2). The uptake was either focal or multifocal in these 8 cases. Abnormal annexin uptake was present in all 7 patients with cortical strokes and in 1 of 5 patients with subcortical strokes (\(P = 0.01\)). Abnormal annexin uptake was significantly and inversely related to the consciousness score on admission (\(r = -0.7, P = 0.02\)). In five of eight patients with abnormal studies, foci of annexin V uptake were also noted contralateral to the affected hemisphere.

2.4. DTPA SPECT imaging

All seven patients who underwent DTPA SPECT showed breakdown of the BBB. The mean DI was 9.9 ± 7.9 (range 3.2–26.3). DTPA ratio was significantly and positively associated with NIH score (\(r = 0.80, P = 0.01\)) and inversely associated with GCS (\(r = -0.89, P = -0.03\)). There was no significant correlation between the AI and the DI (\(r = -0.28, P = 0.54\)). In all cases, image fusion showed only partial overlap between DTPA and annexin V: DTPA was more widely distributed, except in one case (Fig. 2).

3. Discussion

Apoptosis plays a critical role in the pathogenesis of a number of disorders including cerebral and myocardial ischemia, autoimmune and neurodegenerative diseases, epilepsy, traumatic brain injury, organ and bone marrow transplant rejection, and tumor response to chemotherapy and radiation (Belhocine et al., 2002; Blankenberg et al., 1998; Vriens et al., 1998).

Intraneuronal DNA fragmentation, a main feature of the PCD pathway of cell death, has been found in animal models of brain ischemia and in human post-stroke tissue (Chen et al., 1997). Two main pathways have been identified as playing a role in the PCD pathway after stroke. The caspase-dependent pathways are characterized by expression and activation of caspase-3 (Chen et al., 1998) and overexpression of X-chromosome-linked inhibitors of caspase-3 and caspase-7 (Namura et al., 1998). This pathway is rapidly activated after ischemia in the penumbral area. The caspase-independent pathway is based on activation of apoptosis-inducing factor (AIF) and its release from damaged mitochondria (Zhang et al., 2002). It seems that the latter pathway is also activated post-ischemia in the penumbral region.

Regardless of the particular trigger, all cells, including neurons, rapidly redistribute phosphatidylserine (PS, a anionic constitutive membrane lipid) from the inner to outer leaflet of
the plasma membrane lipid bilayer shortly after the onset of apoptosis (van Heerde et al., 1995). It is at this point that annexin V, an endogenous human protein, recognizes and binds to membrane-bound PS that is selectively expressed on apoptotic cells (Zwaal and Schroit, 1997). Due to its high affinity for PS expressing cells, annexin V has been labeled with fluorescein dyes for the in vitro detection of apoptotic neurons, fibroblasts, carcinomas, lymphomas, as well as hematopoietic, endothelial, smooth muscle, and embryonic cells (O’Brien et al., 1997).

Recent investigations have shown that pathophysiologic stresses on neurons and other cells can also cause low to intermediate levels of PS exposure without being associated with an irreversible commitment to cell death in which much higher levels of PS would be expected (Geske et al., 2001; Hammill et al., 1999). These lower levels of PS expression have been linked to a newly described endocytic pathway that is activated by annexin V (Kenis et al., 2004). Annexin V binding to sites of PS exposure reverses the movement of PS containing domains within the cell membrane from blebbing (evagination) into invagination.

This results in formation of annexin V containing intracellular vesicles, thereby permitting the trafficking of this protein within apoptotic cells. Furthermore, this annexin V pump mechanism appears to exist in vivo in cardiomyocytes that express PS reversibly on their surface in the setting of mild ischemia/reperfusion-related stress. This endocytic pump may be the mechanism by which radiolabeled and fluorescent forms of annexin V gain access to the brain through the BBB and neuronal membranes in experimental animal models of cerebral ischemia (D’Arceuil et al., 2000; Mari et al., 2004).

Radiolabeled forms of annexin V have also been used in vitro and more recently in vivo for the detection of apoptotic cells and tissues (Blankenberg et al., 1998). Organ uptake values and absorbed doses indicate that Tc-99m-annexin is accumulated mainly in the kidneys and to a lower degree in the liver and spleen (Kemerink et al., 2001). Since uptake in the normal brain is negligible, it is an ideal tracer for imaging apoptosis in this organ.

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The figure illustrates Annexin V in stroke. (A) Annexin V brain SPECT in a patient with a right peri-ventricular stroke, showing a wider distribution of annexin (top, arrows) compared to the CT findings (bottom). (B) Another patient with a cortical stroke localized to the left MCA region shows extensive stroke on CT (bottom) with selective uptake of annexin in the same region (top, arrows), crossing the midline.

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In this study, the uptake of annexin V was evident not only in the ischemic hemisphere but also in the contralateral “normal” hemisphere, suggesting contralateral neuronal injury and/or stress after acute stroke in a similar fashion as that observed in experimental animal models (D’Arceuil et al., 2000; Mari et al., 2004). Significant increases in the transcription of a number of genes within both hemispheres and striatum in animals suffering from unilateral MCA ischemic injury have been noted by previous investigators (Kim et al., 1998; Rohatgi et al., 2004). A genetic link was suggested for contralateral brain injury mediated via diaschisis, possibly induced from the ischemic cortex and striatum through a polysynaptic transneural pathways. Polysynaptic pathways may also be activated with high levels of excitatory neurotransmitter release causing contralateral stress with subsequent PS exposure on neurons outside the actual site of infarct.

These observations further support the idea that annexin V imaging of PS expression in the human brain may define tissues at risk for cell death that may recover or be amenable to therapeutic intervention. Because the actual cell loss in these patients is gradual in contrast to the immediate irreversible damage caused by severe prolonged ischemia, there may be a therapeutic window to inhibit (or reverse) early apoptosis with pharmacologic blockade.

One potential downside to the use of HYNIC-annexin V is the relatively high nonspecific uptake of the tracer in the calvarial bone marrow that may limit its usefulness in peripheral lesions of the posterior fossa and skull base. SPECT/CT or fusion imaging with separate SPECT and image data sets may help but are not likely to completely solve the problem of confounding calvarial bone uptake as spatial registration and image fusion are rarely ideal in the clinical situation. Possible solutions involve the use of alternative forms of radiolabeled annexin V such as annexin V-128 (Tait et al., 2005). This recombinant mutant of human annexin V can be site-specific labeled $^{99m}$Tc on a five-amino acid tag. Once labeled, annexin V-128 has a markedly lower (less than 10%) nonspecific renal cortical and bone marrow uptake of tracer as compared with HYNIC-labeled annexin V. Annexin

![Fig. 2 – Annexin V compared to DTPA distribution. Two patients, one with a left MCA stroke (A) and one with a right MCA stroke (B), showing annexin uptake in the stroke region (top, arrows), with more extensive Tc-99m-DTPA uptake in the corresponding regions (bottom).](image-url)
V-128 however is still under development and has not been reduced to a simple two-step kit for use in humans.

4. Conclusion

This study shows that it is possible to identify in vivo regions of ischemic neuronal injury and neuronal stress using radiolabeled annexin V in patients suffering from an acute stroke. Since apoptosis can be reversed in the early phases of the process, annexin imaging can play a major role in the selection of therapy in the initial period following stroke in adults.

5. Experimental procedures

5.1. Patient population

Twelve consecutive patients (7 male and 5 female; mean age 66, range 36–83 years) with signs and symptoms of an acute stroke were identified and included in the study. Demographic data and risk factors are shown in Table 1. The study was approved by the ethics committee of our institution. All patients or their first degree relatives signed an informed consent form. Included were male and female patients over the age of 18, with first ischemic stroke in the distribution of the middle cerebral artery (MCA). The World Health Organization’s definition of stroke was used (WHO Regional Office for Europe and the European Stroke Council, 1996). Excluded were patients with recurrent strokes of any type or other diseases of the central nervous system.

Each patient underwent a complete neurological examination. The neurological status was scored using NIH scale (Wityk et al., 1994) and Glasgow Coma Score (Sternbach, 2000) on admission. All patients had an infarct in the MCA territory, either cortical (7 patients) or subcortical, in the corona radiata (5 patients, Table 2) based on CT scanning at time of diagnosis. No patient had a basal ganglia stroke as assessed on initial CT scans.

Five additional patients without neurological abnormalities who were studied with annexin V for unrelated medical conditions (e.g., assessment of hip replacement and colon cancer patients) underwent a brain SPECT study to evaluate the physiologic distribution of annexin V in the human brain.

5.2. CT scanning

Each stroke patient underwent 16-slice spiral computed tomography (CT) without contrast material using Somatom Sensation cardiac (Siemens) shortly after admission and prior to annexin V imaging. The CT scans were used to determine the volume of stroke, as previously described (Brott et al., 1989; Mankovsky et al., 1996).

The location of stroke was categorized into three groups: (a) cortical infarction with mixed involvement of gray and white matter; (b) subcortical infarction of the corona radiata with predominant involvement of white matter; and (c) subcortical infarction of the basal ganglia with predominant involvement of gray matter.

5.3. Preparation of 99mTc-HYNIC annexin V for human use

NAS 2020 Kits were generously supplied by Theseus Imaging Corporation, Boston, MA to the Principal Investigator. Human recombinant (rh) annexin V (MW = 35,806) for the kit was

| Table 1 – Clinical scores in patients with acute stroke |
|---------------------------------|-----|-----|-----|-----|-----|-----|-----|
| No. | HTN | DM | CVD | Lipids | Cig | COPD | Renal | Anemia | |
|-----|-----|----|-----|--------|----|------|-------|--------|
| 1   | 0   | 0  | 0   | 0      | 1  | 0    | 0     | 0      |
| 2   | 1   | 0  | 1   | 1      | 1  | 0    | 0     | 1      |
| 3   | 1   | 0  | 1   | 1      | 1  | 0    | 0     | 1      |
| 4   | 1   | 1  | 0   | 1      | 0  | 0    | 0     | 0      |
| 5   | 1   | 1  | 0   | 0      | 1  | 0    | 0     | 0      |
| 6   | 1   | 1  | 1   | 0      | 1  | 1    | 1     | 0      |
| 7   | 0   | 1  | 0   | 0      | 0  | 0    | 0     | 0      |
| 8   | 0   | 0  | 0   | 0      | 1  | 1    | 0     | 0      |
| 9   | 1   | 0  | 0   | 0      | 0  | 0    | 0     | 0      |
| 10  | 1   | 0  | 0   | 0      | 0  | 0    | 0     | 0      |
| 11  | 1   | 0  | 0   | 0      | 0  | 0    | 0     | 0      |
| 12  | 1   | 1  | 0   | 1      | 0  | 0    | 0     | 0      |


<p>| Table 2 – Imaging characteristics in patients with acute stroke |
|---------------------------------|-----|-----|-----|-----|-----|-----|-----|</p>
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>NIH</th>
<th>GCS</th>
<th>Brain territory</th>
<th>Annexin Pattern</th>
<th>Annexin ratio</th>
<th>DTPA ratio</th>
<th>Infarct volume (cm³)</th>
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<tr>
<td>1</td>
<td>36</td>
<td>M</td>
<td>16</td>
<td>6</td>
<td>Rt MCA</td>
<td>Multifocal</td>
<td>15.6</td>
<td>NA</td>
<td>140.6</td>
</tr>
<tr>
<td>2</td>
<td>69</td>
<td>M</td>
<td>17</td>
<td>NA</td>
<td>Lt MCA</td>
<td>Multifocal</td>
<td>2.3</td>
<td>8.4</td>
<td>142</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>M</td>
<td>16</td>
<td>6</td>
<td>Lt MCA</td>
<td>Multifocal</td>
<td>3.8</td>
<td>5.9</td>
<td>145.5</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>M</td>
<td>4</td>
<td>13</td>
<td>Subcortical</td>
<td>Multifocal</td>
<td>2.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>F</td>
<td>6</td>
<td>13</td>
<td>Subcortical</td>
<td>Negative</td>
<td>1</td>
<td>NA</td>
<td>0.56</td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td>M</td>
<td>2</td>
<td>13</td>
<td>Subcortical</td>
<td>Negative</td>
<td>1</td>
<td>NA</td>
<td>23.1</td>
</tr>
<tr>
<td>7</td>
<td>62</td>
<td>M</td>
<td>5</td>
<td>13</td>
<td>Subcortical</td>
<td>Negative</td>
<td>1</td>
<td>NA</td>
<td>0.7</td>
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<tr>
<td>8</td>
<td>40</td>
<td>M</td>
<td>9</td>
<td>13</td>
<td>Subcortical</td>
<td>Negative</td>
<td>1</td>
<td>3.2</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>74</td>
<td>F</td>
<td>8</td>
<td>11</td>
<td>Lt MCA</td>
<td>Multifocal</td>
<td>1.8</td>
<td>7.5</td>
<td>87.4</td>
</tr>
<tr>
<td>10</td>
<td>81</td>
<td>F</td>
<td>14</td>
<td>9</td>
<td>Lt MCA</td>
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<td>3.8</td>
<td>13.4</td>
<td>29.1</td>
</tr>
<tr>
<td>11</td>
<td>78</td>
<td>F</td>
<td>13</td>
<td>9</td>
<td>Lt MCA</td>
<td>Focal</td>
<td>1.6</td>
<td>26.3</td>
<td>47.6</td>
</tr>
<tr>
<td>12</td>
<td>83</td>
<td>F</td>
<td>17</td>
<td>6</td>
<td>Lt MCA</td>
<td>Focal</td>
<td>6.1</td>
<td>4.4</td>
<td>137.7</td>
</tr>
</tbody>
</table>

* Territory of perfusion abnormality.

b Middle cerebral artery.
produced by expression in Escherichia coli as previously described (Funakoshi et al., 1987). Hydrazine nicotinamide (HYNIC) derivatized annexin V was prepared for later radiolabeling with a 99mTc-tricine precursor complex according to (HYNIC) derivatized annexin V was prepared for later radiolabeling with a 99mTc-tricine precursor complex according to protocols (Larsen et al., 1995; Vanderheyden et al., 2002; Verbeke et al., 2003).

The two-vial kit includes a vial of 0.5 ml (0.25 mg) frozen liquid HYNIC-rh-annexin V conjugate solution (vial 1) and a vial of lyophilized stannous tricine containing approximately 20 mg tricine and 500–650 μg stannous chloride (vial 2). Radiolabeling was conducted at room temperature. 30–50 mCi (0.4–0.6 mCi) of Tc-99m-pertechnetate was added to the HYNIC-rh-annexin V conjugate (vial 1). The lyophilized stannous tricine (vial 2) was reconstituted with 3 ml of sterile saline. Then, an aliquot of 0.3 ml of stannous tricine solution was added to the HYNIC-rh-annexin V vial. The vial was mixed gently, allowed to incubate at room temperature, diluted by the addition of 2 ml sodium chloride for injection, and tested for quality by visual inspection, pH, and radiochemical purity testing with instant thin layer chromatography (ITLC) SG and ACD (acid citrate/dextrose) solution, and pH strips necessary for the quality control of the product. Radiochemical purity of the drug product was verified to be greater than 90% radiochemical purity, as determined by ITLC (ACD) in each dose administered. Each 15 mCi to 20 mCi (555 to 740 MBq) 99mTc-HYNIC-rh-annexin V dose was administered through an indwelling intravenous (IV) line, utilizing the sterile syringe supplied as part of each 99mTc-HYNIC-rh-annexin V kit.

5.4. Annexin V SPECT imaging

SPECT images were obtained in the study group shortly after the CT scan. In two patients, the study was performed less than 24 h after the onset of stroke symptoms and in the remaining 10 patients at 24–48 h after the stroke. A dual head gamma camera (Elscint-Helix, Haifa, Israel) equipped with a pair of low energy, high-resolution collimators was used. Images were acquired in a 128 × 128 matrix at 6° angular steps, with 40 s in each step.

Acquired images were transferred to a dedicated Xeleris workstation (General Electric Medical Systems) for processing. Raw SPECT data were reconstructed using a commercially available Ordered Subset Expectation Maximization algorithm (OSEM; two iterations, ten subsets) and post-filtered using a Butterworth filter (cutoff frequency, 0.5; order, 10.0). The SPECT images were evaluated together with the CT scan.

A quantitative index of apoptosis (AI) was defined as the ratio of mean count per pixel in the infarcted region compared to the mean count per pixel in the contralateral hemisphere, in areas without increased annexin V uptake.

5.5. DTPA SPECT imaging

The integrity of the BBB was evaluated in a subgroup of seven patients available for imaging (6 with cortical and one with subcortical stroke) using DTPA brain scintigraphy at 24 h after the annexin imaging. Each patient in this subgroup received 740 MBq Tc-99m-DTPA, and a SPECT study was performed 2 h later, using the same acquisition parameters as outlined above for annexin imaging. A quantitative index of BBB breakdown (disruption index, DI) was defined as the ratio of mean count per pixel in the infarcted region compared to the mean count per pixel in the contralateral non-affected hemisphere.

Image fusion of the annexin V and DTPA studies was performed with a commercially available software program using a semi-automatic voxel-based algorithm. Both SPECT studies were evaluated based on fused images and on side-by-side evaluation as well.

5.6. Statistical analysis

Analysis of data was carried out using SPSS statistical analysis software (SPSS Inc., Chicago, IL, USA, 1999). Normalcy of distribution of continuous variables was assessed using the Kolmogorov–Smirnov test (cutoff at P = 0.05). Values were compared by each of the comorbidities using the t test for independent samples. Associations between continuous clinical and imaging data were described using Pearson’s correlation coefficients. All tests were two-sided and considered significant at P < 0.05.

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References


